THE JOURNAL

OF

PHARMACOLOGY

AND

EXPERIMENTAL THERAPEUTICS

FOUNDED BY JOHN J ABEL

OFFICIAL PUBLICATION
OF THE AMERICAN SOCIETY FOR PHARMACOLOGY AND
EXPERIMENTAL THERAPEUTICS INCORPORATED

VOLUME 71
1941

BALTIMORE, MARYLAND

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JOHNSON REPRINT CORPORATION
111 Fifth Avenue, New York 3, New York

Johnson Reprint Company Limited
Berkeley Square House, London, W 1

First reprinting, 1963, Johnson Reprint Corporation

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THE TOXICITY OF STRONTIUM AND CALCIUM

VERSA V COLE, BEN K. HARNED AND ROBERTA HAFKESBRING

From the Laboratories of Pharmacology and Physiology Woman's Medical College of Pennsylvania, Philadelphia, Pa.

Received for publication September 14 1940

The close chemical relationship of strontium to calcium and its ability to replace calcium in Ringer Locke solutions (1, 2) led the authors to investigate the relative toxicities of the two ions in rats and mice and to make some observations on the site of action of lethal quantities. Although the literature contains a few data on the toxicities of these ions, comparable dosage-mortality curves are not available for any species.

METHODS

The acetates of strontium and calcium and an equimolecular mixture of the two were administered intravenously to unanesthetized rats and mice and to a few anesthetized rats Each solution was injected in a concentration of 233 millimols per liter at a rate averaging 0.3 millimols per minute through the saphenous vein of the rat, and as fast as a moderate thumb pressure and a 26 gauge needle would permit through the tail vein of the mouse. Twenty animals were used for each point in the curves on toxicity (fig 1)

The cardiac changes in 24 rats equally divided between strontium and calcium, were followed with the electrocardiograph

RESULTS

Strontium acetate proved considerably less toxic than an equimolecular quantity of calcium acetate in mice (fig 1) With an injection of 1 16 millimols per kilogram, the mortality was 5 per cent from strontium but 70 per cent from calcium. The chance that these figures are the same is less than 1 in 10 000 000 Strangely enough the toxicity of the equimolecular mixture of strontium and calcium was slightly greater than that expected by calculating the total concentration of cations as that of the most toxic ion (fig 1) All of the deaths in mice occurred within an hour and most of them within a few minutes.

With rats the difference in toxicity between strontium and calcium was not great and a significant distinction appeared only in the smaller doses Representative of this range is 0 93 millimols per kilogram (fig 1) with a chance of 2 in 1000 that the difference is not significant. There is no signifi

cant difference between the toxicities of strontium and calcium acetates above 1 39 millimols per kilogram in the rat For example, at 1 86 millimols per kilogram the chances that the difference is not significant are 37 in 100 Rats succumbing to strontium died within 70 minutes with an average of 7 3, while those succumbing to calcium died within 12 hours with an average of 70 minutes The chances that this difference is not significant are about 5 in 100 At 50 minutes, 98 per cent of those succumbing to strontium and 84 per cent of those succumbing to calcium were dead When strontium and calcium were combined in equimolecular quantities, the toxicity of the

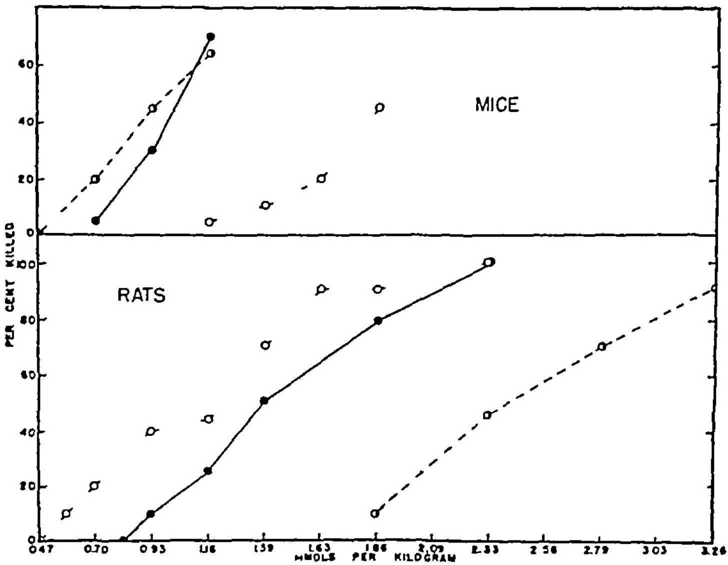

FIG 1 INTRAVENOUS TOXICITY OF STRONTIUM AND CALCIUM
○ Strontium acetate ● Calcium acetate ◑ Strontium and calcium acetates (equimolecular mixture) Each point represents 20 animals

mixture was about equal to that of the strontium component alone, in the range up to a mortality of 85 per cent, a result in marked contrast to that obtained with mice Loesser and Konwiser (3) comparing intravenously injected strontium chloride, bromide and iodide with calcium chloride reported that with rats the M L D for strontium was 1 40 millimols per kilogram and for calcium 1 52

When sodium pentobarbital was administered intraperitoneally in a dose of 30 mgm per kilogram, 10 to 30 minutes before the injection of the strontium or calcium, the toxicity of the former was decreased markedly, while that of the latter was not altered significantly With a dose of 1 86 millimols

TABLE 1

The effect of strontium and calcium on the electrocardiogram of the rat

RAT NUMBER	HEART RATE PER MIN Control	HEART RATE PER MIN Drug†	E. K. G. CHANGES BEFORE RESPIRATORY FAILURE	CAUSE OF DEATH	AUTOPSY FINDING
			Strontium		
1 2, 13 15 16 25 26 28, 29			Slight slowing, no irregularities	Resp. failure	Heart beating regularly
Average	378	334			
5	326		Respiration stopped before record could be taken palpated heart beating regularly	Resp failure	
7	333	125	Slow irregular ventricular rate no fibrillation	Cardiac failure	
12	368	163	Slow ventricular contraction, no irregularities	Resp failure	
Average	373	299			
			Calcium		
4	353	117	Slowing of ventricular rate cessation of ventricular beat. Atrial fibrillation	Cardiac failure	
8	326	326	Heart stopped suddenly before irregularities appeared	Cardiac failure	
9	357	357	Heart stopped suddenly before irregularities appeared	Cardiac failure	
10	288	288	Atrial fibrillation	Cardiac failure	Ventricles contracted
11	211	64	Slow irregular ventricular rate atrial fibrillation	Cardiac failure	Ventricles contracted
17	356	356	Dropped beats block	Resp failure	
18	326	71	Dropped beats block cessation of ventricular activity atrial fibrillation	Cardiac failure	
19	375	258	Cessation of ventricular activity atrial fibrillation	Cardiac failure	Ventricles contracted atria fibrillating
20	312	326	Cessation of ventricular activity atrial fibrillation	Cardiac failure	Ventricles contracted atria fibrillating
21	300	241	Cessation of ventricular activity atrial flutter	Cardiac failure	Ventricles contracted atria fibrillating
22	375	159	Sudden cessation of activity	Cardiac failure	Ventricles fibrillating
23	417	147	Sudden cessation of activity	Cardiac failure	Ventricles fibrillating
Average	333	226			

Rat anesthetized with 30 mgm. per kgm. of sodium pentobarbital intraperitoneally

† 5 0 per cent $Sr(CH_3COO)_2 \cdot \frac{1}{2}H_2O$ or 4.1 per cent $Ca(CH_3COO)_2 \cdot H_2O$ injected continuously intravenously until respiratory or cardiac failure occurred The heart rate represents the average before the onset of irregularities.

per kilogram the fatalities from strontium were 45 per cent with the barbiturate and 90 per cent without it, and the chance that this difference is not significant is less than 1 in 1000 Using an equimolecular quantity of calcium acetate, the fatalities were 95 per cent with the pentobarbital as compared to 75 per cent without it The chances that this difference is not significant are 14 in 100 Convulsions preceded death usually with strontium, but much less frequently with calcium

Electrocardiograms taken from rats given 30 mgm per kilogram of sodium pentobarbital, to facilitate the manipulations, and subsequently strontium or calcium acetate intravenously, disclosed a striking difference in the site of action of lethal doses of these ions A control tracing was taken after the pentobarbital but before the strontium or calcium, and following the injection of 0 7 millimols of strontium or calcium per kilogram, a second tracing was started and continued with the injection of the drug until the death of the animal The results are shown in table 1

With strontium the only consistent change in the cardiac activity was a slight slowing in rate Of the 12 rats studied, only one showed a cardiac irregularity before cessation of respiration Irregularities were not apparent until more than one-half minute after respiration had ceased, these were probably asphyxial and should not be attributed to the drug Autopsies of these rats immediately after cessation of respiration (table 1) showed hearts beating regularly

The electrocardiographic studies following the injection of calcium acetate are in direct contrast to those after strontium Respiratory failure caused death in only one of the 12 rats examined In every other case respiration continued after definite cardiac failure With calcium the cardiac changes were not uniform In several cases there were prolongation of the P-R interval, dropped beats, and block before complete cessation of ventricular activity In the majority of cases defective intraventricular conduction was shown in prolonged Q R S intervals, and in some, a definite shifting of the S-T segment occurred These changes were followed by a cessation of the ventricular beats, and a flutter or fibrillation of the atria Similar effects from calcium were obtained in the rabbit heart by Hoff and Nahum (4) In several cases (table 1) while the electrocardiogram was being taken, definite changes could be seen Then, following cessation of the ventricular beats but while the animal was still breathing, the chest was opened, and examination showed contracted ventricles and fibrillating atria

SUMMARY

1 The relative toxicity of intravenously administered strontium and calcium depends on the species tested In rats the toxicity of strontium exceeds slightly that of calcium in doses producing a mortality less than

60 per cent, but in larger doses there is no significant difference between these ions. In mice strontium is much less toxic than calcium

2 An equimolecular mixture of strontium and calcium acetates in rats exhibits a toxicity equal to that of the most toxic ion regardless of the other ion in the range up to a mortality of 85 per cent. In mice the mixture of the ions exerts a synergistic action producing a mortality slightly higher than that expected by calculating the total concentration of cations as that of the most toxic ion

3 Strontium is partially antagonized by pentobarbital, but calcium is not

4 Electrocardiographic studies showed that strontium produced death by respiratory failure in ninety per cent of the rats, and that calcium produced death by cardiac failure in exactly the same percentage

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THE ACTION OF STRONTIUM AND CALCIUM ON THE UTERUS

BEN K HARNED AND VERSA V COLE

From the Laboratory of Pharmacology, Woman's Medical College of Pennsylvania, Philadelphia, Pa

Received for publication September 14, 1940

The rôle of calcium in uterine contractility attracted the attention of Blair-Bell (1) in 1909 and subsequent experiments, *in vitro* (2, 3, 4) and *in vivo* (5, 6, 7, 8, 9, 10), have established its importance Using isolated uteri from the guinea pig, Van Dyke and Hastings (3) found that variations in all of the physiologically essential ions singly or together altered the response and that changes in the calcium ion concentration produced relatively the greatest change in response Danforth and Ivy (9), employing postpartum dogs and rabbits for *in vivo* studies of uterine activity, observed that the intravenous injection of sodium hexametaphosphate, which removed the ionic calcium, immediately produced complete quiescence even though the uterus had been extremely active prior to the injection The administration of calcium produced a prompt return of uterine activity, and according to these authors the action of calcium and its antagonist was as dramatic and almost as prompt as titrating acid against base Indeed these results indicate that the percentage of calcium in the uterus, amounting to nearly twice that of the bladder muscle, more than twice that of cardiac muscle, and three times that of skeletal muscle (11), is not fortuitous

Although the stimulating action of calcium on the uterus is dramatic under appropriate conditions, its value in the treatment of uterine inertia is very limited, this indicates that its action leaves much to be desired and suggests that a closely related element such as strontium might show an accentuation of the oxytocic properties and possibly find a useful place in therapeutics Many reports on the pharmacodynamic action of strontium have appeared in the literature (12, 13, 14, 15) but with the exception of a few experiments by Boriani (14) its action on the uterus has been neglected Previous studies indicating that strontium and calcium have toxicities of the same order (16) have been confirmed by us (17)

METHODS

The data reported in this paper were obtained with isolated uterine strips of known history, removed from rats, guinea pigs, rabbits and dogs except for the dogs all animals were killed by cervical concussion Two uterine segments, as nearly identical as possible, were immersed in individual 39 cc portions of an oxygenated modified

Locke s solution maintained at 37.5°C and simultaneous kymographic records of the isotonic contractions were made The modified Locke s solution was prepared without calcium and concentrated so that for the other ions 39 cc. contained the salt equivalent of 40 cc of the original Locke s solution thus permitting the addition of calcium or strontium solutions without an objectionable dilution. These solutions were so prepared that 1 cc contained the calcium or strontium chloride equivalent required for 40 cc. of the original Locke s solution (18) whose composition (in millimols per liter) was NaCl 157 3 KCl 5 64 $CaCl_2$ 1 62 $NaHCO_3$ 1 79 glucose 5.55 The bath was maintained between pH 7.5 and 7 7 by passing the oxygen through 1000 cc of 0 75 per cent $NaHCO_3$ The pH of the bath was determined when the solution was removed from the contracting strip and corresponding baths duplicated each other closely Each strip worked with the same leverage (4 8 cm from attachment to fulcrum and 16.5 cm. from fulcrum to writing point) and against the same load (0 40 grams, unbalanced weight from fulcrum to writing point)

The actions of the paired strips duplicated each other with remarkable precision and for the first 25 minutes the contractions resembled those obtained in an unmodified Locke s solution but in the course of 35 minutes the character of the contractions indicated a loss of calcium from the muscle At this time the solutions were replaced with fresh 39 cc. portions of the modified Locke s, and following a 10 to 15 minute interval calcium was added to one bath and strontium to the other When suitable records had been obtained the baths were replaced again by the modified Locke s solution and the calcium and strontium ions were reversed so that each strip was subjected to the action of each ion In some experiments a strip was subjected to the action of only one of the ions however the activity under each ion was always compared with that of the control period for the individual strip Consequently a satisfactory comparison of the ions does not depend upon their action on the same strip

Analyses of the records involved a comparison of (a) changes in tonus and the time relations involved (b) the frequency and uniformity of the contractions and (c) the total height of the contractions per unit of time

RESULTS

In these experiments the action of strontium or calcium consisted of three phases In the first phase either ion led to relaxation from the tetanic contraction which resulted from the pathological increase in the potassium calcium ratio during the washing in Locke s solution containing no calcium (2) When the ions were delicately balanced against potassium the muscle remained inactive for considerable periods of time In the second phase these ions stimulated the muscle to an activity of the normal order and with increased concentrations produced the third phase a tetanic contraction of the uterus

Preliminary experiments showed that the substitution of strontium for calcium increased the frequency but decreased the amplitude of the contractions however a mixture of 4 millimols of strontium to 1 of calcium (abbreviated 4Sr–1Ca) produced contractions whose rate was dictated by strontium but whose amplitude was dominated by calcium Although the number of experiments leading to the adoption of this strontium calcium ratio was not large we believe them to have been sufficient to indicate that the range for optimal efficiency had been attained the data reported in this paper concern

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sium are plotted in figure 1 Concentrations of 0.32 millimols per liter returned the tonus of the strip to that characteristic of its original activity and for the uteri postpartum and also in diestrus further additions up to 1

TABLE 1

Effect of strontium and calcium on the activity of the rat uterus

Seven rats Uteri 24 to 120 hours postpartum

CONCENTRATION OF $CaCl_2$ OR $SrCl_2$	NUMBER OF RATS	NUMBER OF TRIALS	NUMBER OF TRIALS SHOW IN GREATER ACTIVITY		AVERAGE CHANGE IN ACTIVITY IN PER CENT*	
			4Sr-1Ca†	Ca	4Sr-1Ca†	Ca
millimols per liter						
0 32	4	7	5	2	+29	−14
0 64	5	9‡	6	2	+40	+10
0 96	4	5	4	1	+40	+4
1 28	5	6	4	2	+41	+10
1 60	4	4	2	2	+98	+41
2 88 or 3 20	3	3	3	0	+45	+11
Total		34‡	24	9	+293	+62

Five rats Uteri in diestrus

0 32	4	5‡	4	0	−32	−72
0 64	5	5	3	2	+2	−6
0 96	4	5	3	2	+51	+43
1 28	2	2	0	2	+23	+30
1 60	3	3	2	1	+82	+63
2 88 or 3 20	2	2	1	1	+112	+61
Total		22‡	13	8	+238	+119

Four rats Uteri in diestrus

			Sr no Ca		Sr no Ca	
0 32	1	1	1	0	+66	+8
0 64	2	2	2	0	+31	+12
1 60	4	5	2	3	+17	+35
3 20	2	3	0	2	+13	+29
Total		11	5	5	+127	+84

* A comparison of the total height of contractions per minute with that of the control period.

† A mixture of 4 millimols of strontium to 1 of calcium.

‡ In 1 trial strontium and calcium gave identical responses

millimol per liter produced no important changes in tonus However concentrations of calcium above 1.30 millimol per liter[4] produced a marked rise

[4] The calcium content of solutions commonly employed for isolated mammalian muscles ranges from 0.53 to 1 62 millimols per liter with 1 00 millimol per liter representing the usual concentration

Fig 2B

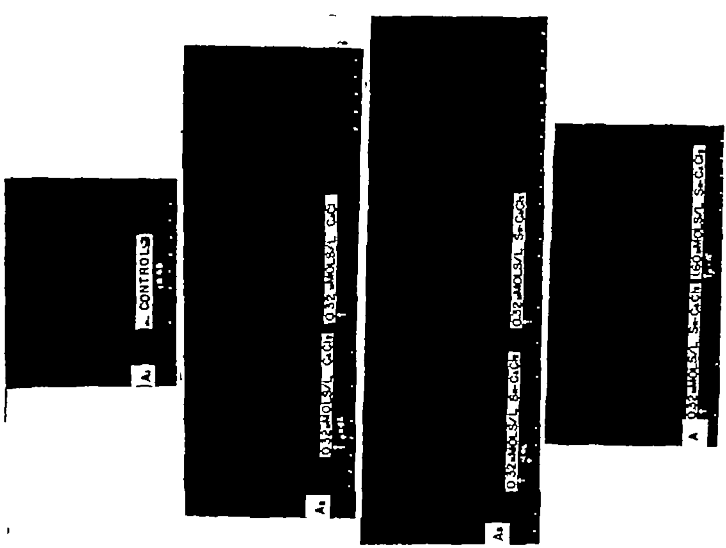

Fig 2A

Legend appears at bottom of opposite page

in tonus actually exceeding that of the original muscle deficient in calcium, Equivalent rises were not produced by 4Sr–1Ca. Up to 1 millimol per liter the calcium or strontium was always added in small increments (fig 2)

The difference between the stimulating action of 4Sr–1Ca and calcium is illustrated by a comparison of the times required for the return of muscular activity following the release of the tetany due to a high potassium calcium ratio (fig 2, A_2 and B_2, A_3 and B_3) An analysis of 28 records showed that the activity always returned faster after 4Sr–1Ca and the average time required was approximately one fifth of that for calcium alone.

Rate and uniformity of contractions Quantitative comparisons of the influence of 4Sr–1Ca on the rate of the maximal contractions show that in 83 per cent of the trials with postpartum uteri the rate is faster in the mixture of strontium and calcium. The averages for concentrations of these ions ranging from 0 64 to 3.2 millimols per liter reveal that 4Sr–1Ca produces a rate 50 per cent greater than that of calcium. For the record of a typical strip see figure 2. Analogous comparisons on uteri in diestrus are qualitatively similar but less striking In 4Sr–1Ca the character of the contractions is more uniform both in height and duration than in calcium and although these differences appear in all concentrations they are exaggerated when these ions exceed 2 millimols per liter

Comparisons of the total heights of the contractions per unit of time would appear to provide the most satisfactory method of assessing the relative effects of 4Sr–1Ca and calcium on uterine activity Such comparisons have been recorded in table 1 where the results are shown as deviations (in per cent) of the activity of the individual strips from the control value The most significant differences between the ions were obtained with uteri 24 to 120 hours postpartum When the concentrations of 4Sr–1Ca or calcium range from 0.32 to 3 2 millimols per liter the averages consistently show an advantage in activity for the mixture. In 70 per cent of the trials individual uteri gave a more satisfactory record of activity in 4Sr–1Ca Some of the comparisons were made on muscles damaged by long-continued immersion in a Locke s solution containing no calcium or by many manipulations, and with

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FIG 2 EFFECTS OF CALCIUM AND STRONTIUM ON THE RAT UTERUS

Rat killed at 9 55, 54 hours postpartum and the two uterine strips (*A* segment from right uterine horn *B*, corresponding segment from left horn) were placed immediately in oxygenated Locke's solution containing no calcium

A_1 B_1 control.

Strips were changed to fresh Locke s solution containing no calcium at 10 53.

A_2 0.32 mMols/liter of $CaCl_2$ added at 11 01 repeated at 11 09

B_2, 0.32 mMols/liter of $(4Sr\text{–}1Ca)Cl_2$ added at 11 00, repeated at 11 08

Strips were placed in fresh Locke s solution containing no calcium at 11 42.

A_3, 0.32 mMols/liter $(4Sr\text{–}1Ca)Cl_2$ added at 12 05, 12 14, 12 53 12 55.

B_3, 0.32 mMols/liter CaCl added at 12 05, 12 14 12 52, 12 54

A_4, 0.32 mMols/liter $(4Sr\text{–}1Ca)Cl_2$ added at 1 08 and 1 60 mMols/liter at 1 16

B_4, 0.32 mMols/liter $CaCl_2$ added at 1 06 and 1 60 mMols/liter at 1 14

The pH of the baths was maintained at 7 6 The time interval was 1 minute The relation of the base line to the recording lever was constant

THE EFFECT OF PARA-AMINOBENZOIC ACID ON THE BACTERIOSTATIC ACTION PRODUCED BY SODIUM PARANITROBENZOATE ON A STRAIN OF STREPTOCOCCUS VIRIDANS

JOHN K MILLER

From the Division of Laboratories and Research, New York State Department of Health, Albany

Received for publication September 17, 1940

During a study of the action *in vitro* of sodium paranitrobenzoate and sodium para-aminobenzoate preparatory to investigating the chemotherapeutic effect of the former substance *in vivo*, unexpectedly variable results were obtained Ten and 100 mgm per cent of the sodium paranitrobenzoate exerted a bacteriostatic action during the first twenty hours' incubation of a strain of Streptococcus viridans in beef-infusion broth (1), but when 200 or 400 mgm per cent of sodium paranitrobenzoate were added to the culture medium this activity was very slight or absent

Since, as reported by Woods (2) and Selbie (3), para-aminobenzoic acid has an inhibitory effect on the bacteriostatic action of sulfanilamide, it appeared possible that part of the paranitrobenzoate was reduced to para-aminobenzoate, which either facilitated bacterial growth or inhibited the bacteriostatic action of the paranitrobenzoate Also, the greater the amount of sodium paranitrobenzoate, the more rapidly the necessary amount of aminobenzoate would be formed and the more effectively the bacteriostatic action would be counteracted To test the validity of this reasoning, the following experiments were performed

intervals by Marshall's method for the sulfonamide compounds (4 5) Diluted broth solutions of sodium para aminobenzoate were used as standards. Recoveries of 90 to 95 per cent or more of the added material were obtained in control determinations

Cultures Serial dilutions in broth were made of a 15-hour infusion broth culture of the *Streptococcus viridans* Duplicate blood-agar poured plates containing 0.000 001 and 0.000 000,1 cc. of the culture were incubated for forty-eight hours at 37 C. and the colonies counted Each colony was considered to represent one microörganism.

To demonstrate the *bacteriostatic activity of the paranitrobenzoate* 0.5 cc of the diluted culture containing 0 000 1 cc (12 000 to 29 000 microörganisms) or 0 000,001 cc. (120 to 290 microörganisms) of the original culture was pipetted into 9.5 cc. amounts of infusion broth containing 10 100 200 and 400 mgm per cent of sodium paranitrobenzoate After 20 27 and 48 hours' incubation at 37 C 0.5 cc of each culture was removed The appropriate dilutions were made in broth blood agar poured plates were made and the colonies counted after 48 hours incubation At the same time samples of the culture fluid were taken for the determination of the presence of any amino compound

To investigate *the inhibitory effect of sodium para-aminobenzoate on the bacteriostasis induced by sodium paranitrobenzoate* a second series of cultures was set up in a similar manner except that to additional control cultures containing 10 100 200 and 400 mgm. per cent of sodium paranitrobenzoate, 0 05 or 0 20 mgm per cent of sodium para-aminobenzoate was added These infusion-broth solutions were inoculated with similar numbers of microörganisms, incubated and the amount of growth and of amino compound determined in the same manner and at the same intervals as in the previous experiment.

Finally *to determine whether under the conditions of the test, para-aminobenzoate facilitated the growth of the strain*, the rate of growth of strain No 40108 in infusion broth containing 0 05 and 0.20 mgm. per cent sodium para-aminobenzoate was compared with the control cultures in infusion broth alone. The number of microörganisms introduced was in the range used in previous experiments. After 6 12 and 24 hours incubation 0.5 cc. of culture was diluted in broth, and blood-agar poured plates were made Samples for determination of the amino compound in the cultures were also taken at the same intervals

RESULTS

The effect of 10 100, 200, and 400 mgm per cent of sodium paranitrobenzoate on the growth of this Streptococcus viridans in infusion broth is shown in table 1

A definite inhibition of growth was effected by 10 and 100 mgm. per cent of sodium paranitrobenzoate which was more marked when the tubes were examined after 20 hours incubation but was still apparent after 27 hours incubation. In those tubes containing 200 mgm per cent, a possible slight bacteriostatic activity was noted in the tubes inoculated with the smaller number of microörganisms With 400 mgm per cent of the drug inhibitory action was questionable or absent.

Tests for the presence of an amino compound in the cultures indicated that, within the limits of the test by Marshall's method, none was present in the tubes in which bacteriostatic action was most marked. However, with increase in bacterial multiplication, amino compound was detectable and occurred in greater concentrations as growth continued.

mgm. per cent of sodium para-aminobenzoate were added. There was no demonstrable difference in the rate of growth and, within the first twenty-four hours, there was no increase in the amount of amino compound.

DISCUSSION

Absence of growth-stimulating activity by para-aminobenzoic acid for Streptococcus hemolyticus has been noted by Woods (2). Likewise, in the above experiments para-aminobenzoic acid did not affect the growth activity of this strain of Streptococcus viridans. However, the addition of 0.05 mgm. per cent of sodium para-aminobenzoate was found to interfere with the bacteriostatic action of the sodium paranitrobenzoate on this same strain. An amino compound was formed in cultures containing the

TABLE 3

Effect of sodium para-aminobenzoate on the growth of a strain of Streptococcus viridans

INOCULUM NUMBER OF STREPTOCOCCI	PARA AMINO BENZOATE	PERIODS OF INCUBATION AT 37 C					
		6 hours		12 hours		24 hours	
		Colonies per cc.	Amino compound	Colonies per cc.	Amino compound	Colonies per cc.	Amino compound
	mgm per cent		*mgm per cent*		*mgm per cent*		*mgm. per cent*
205		15,200	0.00	47,500,000	0.00	345,000,000	0.00
20,500		2,080,000	0.00	350,000,000	0.00	235,000,000	0.00
205	0.05	23,700	0.06	51,000,000	0.05	280,000,000	0.05
20,500	0.05	1,440,000	0.08	300,000,000	0.05	300,000,000	0.05
205	0.20	21,200	0.21	42,300,000	0.20	260,000,000	0.20
20,500	0.20	2,745,000	0.23	420,000,000	0.20	280,000,000	0.20

* Colony count in blood agar—one colony considered to represent one microörganism.

lesser concentrations of paranitrobenzoate alone and its presence was noted when multiplication of the microörganism increased. Also, in the presence of the greater concentrations of paranitrobenzoate, 200 and 400 mgm. per cent, more rapid production of amino compound was noted. Thus, the failure of these latter concentrations to exhibit the bacteriostatic action shown by 10 and 100 mgm. per cent of paranitrobenzoate may possibly be attributed to this effect.

The amount of amino compound formed in the cultures containing paranitrobenzoate was not enough to reduce the amount of paranitrobenzoate to an ineffective level, particularly in those containing 100 mgm. per cent of paranitrobenzoate where the maximum amino compound was only 1.5 mgm. per cent. Since para-aminobenzoate alone did not affect the growth activity of this strain, it was then necessary to conclude that the amino compound

added to cultures with paranitrobenzoate interfered with the mechanism through which the paranitrobenzoate exerts its bacteriostatic action on this strain of Streptococcus viridans.

In addition, it has been shown that a culture of this strain in broth alone produced some diazotizable substance after 48 hours incubation. Also in cultures containing paranitrobenzoate, the formation of the amino compound occurred more rapidly and in larger quantities in those cultures inoculated with the greater numbers of streptococci.

Further studies are in progress including investigations of the sulfonamide compounds and of other strains of Streptococcus viridans.

SUMMARY

In these experiments 10 and 100 mgm. per cent of sodium paranitrobenzoate had a temporary bacteriostatic effect on a strain of Streptococcus viridans whereas with 200 and 400 mgm per cent this effect was absent or slight. With increase of bacterial multiplication, measurable amounts of amino compound detectable by diazotization were present in the cultures This amino compound was formed in larger amounts with the higher concentrations of paranitrobenzoate and with the larger inocula of bacteria.

The bacteriostatic action of the paranitrobenzoate was prevented by the addition of small amounts, 0 05 mgm. per cent of sodium para-aminobenzoate to cultures.

Para-aminobenzoate alone had no demonstrable effect on the growth activity of this strain of Streptococcus viridans

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mgm per cent of sodium para-aminobenzoate were added There was no demonstrable difference in the rate of growth and, within the first twenty-four hours, there was no increase in the amount of amino compound

DISCUSSION

Absence of growth-stimulating activity by para-aminobenzoic acid for Streptococcus hemolyticus has been noted by Woods (2) Likewise, in the above experiments para-aminobenzoic acid did not affect the growth activity of this strain of Streptococcus viridans However, the addition of 0 05 mgm per cent of sodium para-aminobenzoate was found to interfere with the bacteriostatic action of the sodium paranitrobenzoate on this same strain An amino compound was formed in cultures containing the

TABLE 3

Effect of sodium para-aminobenzoate on the growth of a strain of Streptococcus viridans

INOCULUM NUMBER OF STREPTO-COCCI*	PARA AMINO-BENZOATE	PERIODS OF INCUBATION AT 37°C.					
		6 hours		12 hours		24 hours	
		*Colonies per cc.	Amino compound	Colonies per cc.	Amino compound	Colonies per cc.	Amino compound
	mgm per cent		*mgm per cent*		*mgm per cent*		*mgm per cent*
205		15 200	0 00	47,500,000	0 00	345,000,000	0 00
20,500		2,080,000	0 00	350,000,000	0 00	235,000,000	0 00
205	0 05	23 700	0 06	51,000 000	0 05	280,000,000	0 05
20 500	0 05	1,440,000	0 08	300 000,000	0 05	300,000,000	0 05
205	0 20	21,200	0 21	42,300,000	0 20	260,000,000	0 20
20,500	0 20	2,745,000	0 23	420,000,000	0 20	280,000,000	0 20

* Colony count in blood agar—one colony considered to represent one microörganism.

lesser concentrations of paranitrobenzoate alone and its presence was noted when multiplication of the microörganism increased Also, in the presence of the greater concentrations of paranitrobenzoate, 200 and 400 mgm per cent, more rapid production of amino compound was noted Thus, the failure of these latter concentrations to exhibit the bacteriostatic action shown by 10 and 100 mgm per cent of paranitrobenzoate may possibly be attributed to this effect

The amount of amino compound formed in the cultures containing paranitrobenzoate was not enough to reduce the amount of paranitrobenzoate to an ineffective level, particularly in those containing 100 mgm per cent of paranitrobenzoate where the maximum amino compound was only 1 5 mgm per cent Since para-aminobenzoate alone did not affect the growth activity of this strain, it was then necessary to conclude that the amino compound

frigeration (b) "*ficin*"—a cream-colored, water-soluble, amorphous powder containing from 1 to 2 per cent of benzoic acid prepared by filtration and drying of the above-mentioned raw latex and having in one gram the proteolytic activity of approximately 5 cc. of the raw latex. When kept in evacuated ampoules, ficin showed negligible loss of activity throughout the two years of this investigation. Control experiments performed by administering ten times the maximum amount of sodium benzoate present in this material and by giving material from which the preservative was removed showed that the sodium benzoate did not affect the toxicologic findings.

ORAL TOXICITY

The acute toxicity of ficin and leche when taken by mouth was determined in mice, rats, rabbits guinea pigs, cats, and dogs. Ficin was used in aqueous solutions in concentrations of from 10 to 50 per cent, while leche was used undiluted The material was administered perorally to mice rats, and guinea pigs through a No 18 gauge metal cannula and to rabbits cats and dogs through a rubber catheter The animals were observed for a period of 2 consecutive days Deaths within twenty four hours after administration were recorded as "acute those occurring after this time as 'late deaths.

Experiments in mice and rats. In this series a total of 945 mice and 345 rats were used The average weight of the mice was 20 grams that of the rats 150 grams. Ficin was given in doses of from 4 to 20 grams per kilogram and leche in doses of from 25 to 50 cc. per kilogram.

The possibility that the toxicity of ficin may be more dependent on the concentration at the site of contact with the gastrointestinal mucosa than on the total amount administered was first investigated. Varying amounts of ficin in 12 different concentrations ranging from 4 to 75 per cent were fed to mice and rats. No significant differences due to concentration were found. With a total dose of 12 grams per kilogram the mortality was 48 per cent for the 25 per cent concentration and 54 per cent for the 50 per cent concentration, using 50 mice in each experiment.

After these preliminary experiments the complete toxicity range for mice and rats was determined in the usual manner using 20 animals per dose level. The results are shown in figure 1 in which the leche is recorded in terms of ficin proteolytic activity (1 gram ficin corresponding to 5 cc. leche) The somewhat greater toxicity of leche is probably due to the presence in leche of other toxic principles.

Sub-lethal doses of leche and ficin caused dysentery of varying severity, with bloody stools at the higher dose levels. With fatal doses the animals became prostrated and died within 6 to 24 hours after administration Animals which survived the first 24 hours recovered in almost all cases. There was no significant difference in the acute and delayed toxicity

Experiments in guinea pigs and rabbits. As seen from table 1 doses above

3 grams per kilogram for guinea pigs and 5 grams per kilogram for rabbits were fatal, indicating a considerably greater sensitivity in these species than in mice and rats

Experiments in cats and dogs Six cats and 30 dogs were fed with ficin solutions ranging in concentrations from 10 to 50 per cent or with gelatin capsules filled with dry powder Doses which were sufficiently large to exert an anthelmintic effect were well tolerated but doses of 1 0 gram per kilogram or above frequently produced vomiting and diarrhea from which the animals recovered in one to two days Since vomiting could not always be prevented by a preliminary administration of chlorobutanol (1 5 to 2 5 grams per kilogram), an accurate evaluation of the results, especially the determination of the lethal dose, was not possible

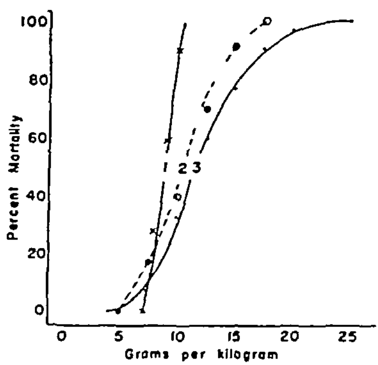

FIG 1 PERORAL TOXICITY OF LECHE AND FICIN IN MICE AND RATS
1 Leche in mice 2 Ficin in rats 3 Ficin in mice

In view of Thomen's findings (5) that several small doses of ficin repeated over a period of several days were as effective therapeutically as one single large dose and less likely to produce undesirable side effects, particularly vomiting, the *cumulative toxicity* of ficin was studied in rats and guinea pigs In experiments conducted in 198 animals, it was found that subdivision of a given amount of ficin into smaller doses resulted in a lowered mortality Thus, in guinea pigs, 1 6 gram per kilogram divided into 4 equal daily doses resulted in 80 per cent mortality, whereas 1 5 gram per kilogram given in 7 doses caused no deaths Similar results were found with rats (table 2) The results indicate that larger total doses of ficin may be safely administered if subdivided

Pathological findings At autopsy severe changes in the gastrointestinal tract were found, ranging from severe inflammatory reaction to erosion At the higher dose levels disintegration of the stomach and intestines frequently

TABLE 1

SPECIES	ANIMAL		DOSE	LENGTH OF SURVIVAL	REMARKS
	Number	Weight			
			grams/kgm.		
Guinea pig	1	590 grams	3 0	8 hours	Congestion of stomach and intestines
	2	570 grams	3 0	<24 hours	Congestion of stomach and intestines
	3	540 grams	3 0	<40 hours	Congestion of stomach and intestines
	4	510 grams	0 80	Indefinite	
	5	490 grams	0 80	Indefinite	
	6	550 grams	0 60	Indefinite	
	7	560 grams	0 60	Indefinite	
	8	720 grams	0 60	Indefinite	
	9	780 grams	0 60	Indefinite	
Rabbit	1		5 0	<16 hours	
	2		5 0	<16 hours	
	3		5 0	<16 hours	
	4		5 0	<16 hours	
	5	3 0 kgm.	5 0	Indefinite	Organs appeared normal
	6	2 9 kgm	4 0	<16 hours	Blood in intestines liver kidney and lungs congested
	7	3 3 kgm.	3 0	Indefinite	
	8		3 0	Indefinite	
	9		3 0	Indefinite	
	10	2 9 kgm.	2 0	Indefinite	
	11		2 0	Indefinite	

TABLE 2

NUMBER OF RATS	DOSE OF FICIN				MORTALITY
	Grams/kgm.	Number of doses per day	Number of days	Total	
				grams/kgm.	
10	20	1	1	20	100
10	10	1	2	20	80
10	5	1	4	20	10
10	2	1	10	20	0

occurred, with subsequent partial digestion of the liver spleen pancreas and kidneys. Histological examination frequently showed a peculiar perifollicular reaction in the spleen varying from fibrin deposits to leucocytic infiltra

tion of this problem In view of the protein nature of ficin it seemed feasible to use for this problem the method of sensitization to anaphylactic shock. In more than 50 guinea pigs, it was found that animals which had been intravenously sensitized with small doses of ficin occasionally responded to its reinjection after a 10 day rest period with anaphylactic symptoms, though this was never observed in animals in which the sensitization had been attempted by daily peroral administration of ficin for 7 days Unfortunately, the positive results after intravenous sensitization were so irregular that it was impossible to attach any particular significance to these findings

Another indication contrary to the assumption that ficin is absorbed from the intestinal tract may be taken from experiments in which its effect on the blood picture was studied In these experiments the peroral administra-

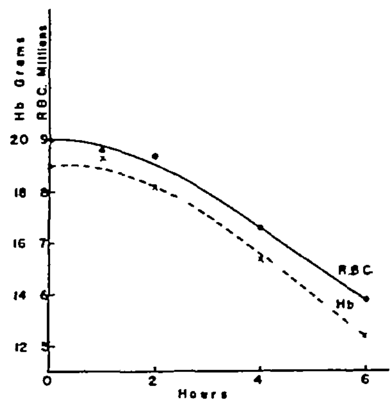

FIG 2 EFFECT OF AN INTRAVENOUS INJECTION OF FICIN IN RATS (50 MGM PER KILOGRAM)

———, erythrocyte count, - - - - -, hemoglobin concentration

tion of doses of ficin 400 times larger than those which produced definite reduction of erythrocyte count and hemoglobin level upon intravenous injection (fig 2) failed to show any effect

Kidney and liver function tests seem likewise to indicate that no absorption of ficin takes place after peroral administration In these experiments, the influence of ficin upon the liver function was determined using the bromosulphthalein test of Rosenthal (6) After normal values had been established in several control runs, 20 per cent ficin solution was given orally for 4 consecutive days in a dose of 0 5 gram per kilogram Daily tests during and after the period of ficin administration showed no variation in the liver function

The effect of ficin upon the kidney function was investigated in a group of 80 rats by comparative diuresis experiments The changes in specific gravity,

volume and chemical composition (albumen blood) of urine following administration of 5 cc. of water per 100 grams body weight was measured at 30 minute intervals. After satisfactory controls were established, 30 per cent ficin solution was given in doses of 0 5 gram per kilogram and 3 grams per kilogram followed 2 hours later by the usual administration of water Diuresis experiments showed changes with the smaller dose In experiments with the larger dose, 60 per cent of the rats showed symptoms of a profuse bloody diarrhea within the next few hours and died, although this dose is normally well tolerated It would appear that the relatively large amount of water which followed an otherwise harmless dose of ficin is responsible for this increase in toxicity

On the basis of the foregoing experiments it does not seem likely that the pathologic changes observed after peroral administration of ficin are due to the specific action of active Ficus proteinase. One of the explanations may be that active ficin has penetrated through a rupture in the intestinal wall and came into direct contact with other organs while the animal was still alive

DISCUSSION The peroral administration of proteolytic enzymes for anthelmintic purposes is based on the assumption that they attack the proteins in the integument of intestinal parasites before damaging the intestinal wall of the host. Such an assumption would seem to be justified not only by the high specificity of enzymes in general, but also by the fact that this same enzyme ficin, readily attacks certain intestinal parasites such as Ascaris and Trichocephalus, whereas it is almost ineffective against others such as Taenia.

Our experiments have shown that ficin may severely injure the intact intestinal mucosa and subsequently cause death, if left in contact for a sufficient length of time and in a sufficiently large quantity and high concentration However the wide use of leche de higueron in Central and South America as an anthelmintic and our own finding of ficin as a highly effective and safe anthelmintic for dogs indicate that its safety margin is very wide

The results of our experiments, on apparently healthy animals give no information regarding the possible complications brought about by the use of ficin in patients with intestinal mucosa already damaged, as through ulcerative processes, etc. A further indication of the increase in toxicity of ficin in animals with damaged intestinal mucosa was given by Lamson, Brown, and Ward (7) who found that the toxicity of ficin was greatly increased if it was given two or three hours after a peroral dose of hexylresorcinol which itself causes a temporary irritation [3]

[3] We are informed that experiments on the comparative toxicity of ficin in normal animals and in animals with damaged intestinal mucosa are being undertaken by Dr E. C Faust and his coworkers in Tulane University and will reported in the near future

Although practical use and clinical observations indicate a great therapeutic latitude in the use of ficin, its marked proteolytic properties—which surpass those of most of the generally available enzymes of this nature, such as papain—suggest caution in its use This necessity is illustrated by our experiments in which a trace of dry ficin applied for less than half a minute to the eye frequently resulted in an irreparable damage of the cornea and by the fact that intravenous injection of as little as 25 mgm caused death within a few minutes

In addition to the experiments reported in this paper, which were all performed with a uniform material, we had an opportunity to test a considerable number of ficus enzymes prepared by various procedures and of different origins and can fully confirm statements in the literature regarding great variation in the potency of these agents It would therefore seem that all ficin preparations used for therapeutic purposes should be biologically standardized and that the dosage should be based on units of proteolytic activity

SUMMARY

(1) The peroral and intravenous toxicity of ficin was determined in mice, rats, guinea pigs, rabbits, cats, and dogs A considerable variation in sensitivity among different species was observed The L D 50 for rats and mice following oral administration is about 10 grams per kilogram and in rabbits and guinea pigs about 5 grams per kilogram The toxicity following intravenous administration (50 to 100 mgm. per kilogram) is in contrast to the low toxicity by the oral route

(2) The toxicity of ficin depends primarily upon the total amount of drug administered and is practically independent of the concentration

(3) The toxicity of a given dose of ficin can be reduced by subdivision into smaller doses and repeated administration

(4) The signs following sublethal doses consist of vomiting, bloody diarrhea, and general prostration Upon autopsy there is severe irritation of the gastrointestinal tract ranging from inflammatory reactions to erosions

(5) Parenteral injection of ficin causes severe tissue damage This is also observed on topical application of ficin to wounds or denuded skin and particularly to the conjunctival surfaces

(6) Repeated daily peroral administration of ficin does not alter the outcome of liver and kidney function tests

(7) Intravenous injection of small doses of ficin reduces the erythrocyte count and prolongs markedly the blood clotting time

(8) On the basis of sensitization to anaphylactic shock there is no evidence of absorption of ficin from the intact gastrointestinal tract

Acknowledgments Our thanks are due to Dr W Antopol for the pathological and histological examinations and Mr O Graessle for valuable technical assistance.

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A COMPARISON OF THE INFANTILE AND ADULT RAT METHODS FOR THE ASSAY OF THE OESTRUS-INDUCING GONADOTROPHIC SUBSTANCE

R. D H HEARD

From the Connaught Laboratories, University of Toronto

Received for publication September 20, 1940

In an earlier communication (Heard and Winton (1)) the use of the dietary anoestrous adult rat as a test object for the estimation of gonadotrophic activity was reported This method of assay, based upon the vaginal reaction to gonadotrophic substance administered during the period of experimental anoestrus, proved most satisfactory and achieved a high degree of accuracy

The long standing and common use of the infantile rat for the same purpose makes desirable a direct comparison of the two tests To this end, dosage-response curves for the 21-day-old rat of the same strain have been constructed under like experimental conditions A comparison of these data with those previously reported for the adult animal clearly shows that the latter test object is the more sensitive and that greater accuracy is attained by its use

MATERIAL AND METHODS

The standard *pregnancy urine extract* and the *statistical methods* and *notation* used were those formerly described (1)

Animals All rats were drawn from Connaught Laboratories' homozygous stock (previously designated as strain C) Since the sensitivity of the infantile animal and the slope of the dosage-response curve is influenced more by the age than the body weight of the test object (Chapman (2)), the first-mentioned variable was held constant Only young females 21 days of age at the time of the first or single injection were used, the average weight was 45 to 50 grams

Methods of administration To conform with the usual practice of spreading the total dose over a period of several days, and with the single injection technique which was proved to be effective in the adult rat test, both procedures were followed The divided dose was given once daily for three days For each test a stock solution approximately 20 mgm per 100 cc) was prepared on the first day, adjusted to pH 7 and stored at −2°C throughout the administration period The appropriate dilutions with 0 9 per cent saline were then made daily *immediately* preceding injection In all cases the volume administered was maintained constant at 0 5 cc

Recording of vaginal smears and criteria of oestrus With both methods of administration smears were recorded (when necessary by forcible opening of the vagina) once (5 p.m) on the third day after the first or single injection, twice (9 a m and 5 p m)

on the fourth and fifth days, and once (9 a.m) on the sixth day The previously stated criteria of oestrus and evaluation of the smears were rigidly adhered to

DOSAGE-RESPONSE CURVES FOR THE INFANTILE RAT

Since the number of 21-day-old females available for each test was restricted to a maximum of twenty, all were injected with the same amount of the standard preparation The dosage-response curves are therefore constructed from a number of observations each made on a different day While the slope of the curve obtained from data combined in this way may

TABLE 1

Induction of vaginal oestrus in 21-day-old rats following the administration of the total dose of the standard preparation in three portions

AMOUNT GIVEN	NUMBER OF ANIMALS	NUMBER OF POSITIVES	RESPONSE
μ grams			*per cent*
4 61	10	2	20 0
4 69	14	½	3 6
6 12	10	1	10 0
	10	2	20 0
	11	2	18 2
6 30	9	½	5 6
7 52	8	4	50 0
	8	1½	18 8
	10	6½	65 0
	18	3½	19 4
8 92	9	5	55 6
	12	4½	37 5
	10	4½	45 0
10 35	9	4½	50 0
	15	9	60 0
	12	6½	54 2
11 52	15	11	73 3
11 75	11	9	81 8
12 12	9	8½	94 4
12 67	14	9	64 3
14 00	20	16	80 0

be slightly lower than the true value owing to fluctuation in sensitivity from day to day it is clearly inadvisable to administer more than one dosage at each test when the available number of test objects is limited The error so introduced is in any case small (see Gaddum (3)) indeed with the adult rat no difference was observed (1) between the value of the slope computed from data combined directly in this fashion and that arrived at by the method of Morrell and Chapman (4) in which the curves obtained at each test were united with the elimination of variation in position.

Divided dose The total dose was given in three portions to twenty-one

groups of anımals (table 1), the resultıng dosage-response curve ıs shown ın figure 1 The upper scale of abscıssa sets out the dose ın terms of the amount requıred to ınduce oestrus ın 50 per cent of the anımals ınjected (ı e the O D 50), ıt thus represents a scale of "rat unıts" applıcable only to anımals of thıs straın and under the partıcular condıtıons outlıned The correspondıng regressıon lıne ıs ıllustrated ın figure 2 There ıs unıform gradatıon of effect accordıng to dose, and the χ^2 test ındıcates agreement well wıthın the lımıts of samplıng error between the experımental data and the lıne fitted to them ($\chi^2 = 16\,8$, $n = 17$, whence $P = 0\,55$)

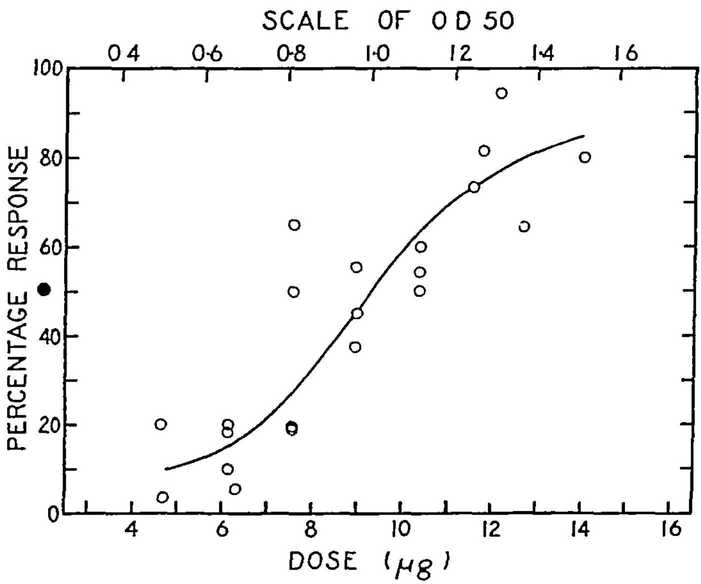

FIG 1 DOSAGE-RESPONSE CURVE FOR 21-DAY-OLD RAT (DIVIDED DOSE)

Sıngle ınjectıon Irregular results were obtaıned wıth nıneteen groups of ınfantıle rats to whıch the total dose was gıven ın one portıon (table 2) The magnıtude of the group varıatıon ın sensıtıvıty does not permıt the dırect fittıng of a satısfactory curve relatıng dose to effect, and clearly emphasızes the fact that the rate of absorptıon must be delayed by subdıvısıon of the dose over a perıod of at least three days ın order to obtaın unıform responses wıth thıs test object By combınıng the responses of those groups gıven the same dosage ınto a sıngle percentage, as ındıcated ın table 2, a reasonable characterıstıc curve was obtaıned (fig 2), but even so the dıscrepancy between these cumulatıve observatıons and the fitted lıne ıs slıghtly

greater than would be expected from chance sampling alone ($\chi^2 = 10.29$, $n = 4$ whence $P = 0.04$) The curve is significantly flatter than that pertaining to the divided dose (fig 2 and table 3)

For practical purposes the single injection method of administration is worthless, the data are useful only for comparison with those obtained under the same conditions with the adult animal

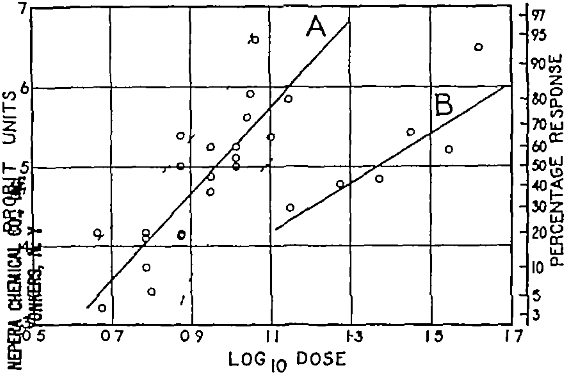

Fig 2. Regression Lines for 21 day-old Rat

Curve A (divided dose) $Y = 4.953 + 5.45 (X - 0.954)$ the dotted lines indicate the zone of error corresponding to ± 2 standard deviations for 11 6 animals (i.e. the average number in each group—see table 1), computed from the usual formula, S.D $= \sqrt{(PQ/N)}$ where P = percentage response, $Q = 100 - P$ and N = number of animals.

Curve B (single injection) $Y = 5.109 + 3.14 (X - 1.399)$

COMPARISON OF THE INFANTILE AND ADULT RAT TESTS

The first and possibly most marked difference noted between the two methods of assay was in the time of duration of the period of induced oestrus. In the dietary anoestrous adult animal cornification persists for 24 to 48 hours following a single injection of gonadotrophic substance and hence vaginal smears need be recorded only once each day in order to include all positive reactions. Little or no difficulty was encountered in evaluating the response as the positive reactions were characterized by the complete disappearance of leucocytes in all but a few cases. With the infant animal however the period of cornification lasts only a few hours even when the total dose is spread over three days and is dependent to a great extent upon the

strength of the stimulus In the present work, observations of the vaginal contents were made daily at 9 a m and 5 p m The number of "doubtfully positive" smears obtained was relatively great which suggests that the interval between 5 p m and 9 a m is too long, but it is inconvenient to examine

TABLE 2

Induction of vaginal oestrus in 21-day-old rats following a single injection of the standard preparation

AMOUNT GIVEN	NUMBER OF ANIMALS	NUMBER OF POSITIVES	RESPONSE
μ grams			*per cent*
14 1	15	4	26 7
	12	4	33 3
	27	8	29 7
18 8	7	1½	21 4
	18	9	50 0
	9	3½	38 9
	34	14	41 2
23 5	15	6	40 0
	15	6	40 0
	10	3	30 0
	8	6	75 0
	48	21	43 7
28 2	7	4½	64 3
	15	10½	70 0
	14	9	64 3
	36	24	66 7
35 2	8	6½	81 3
	10	7	70 0
	7	3½	50 0
	8	6	75 0
	10	2	20 0
	43	25	58 2
41 7	16	15	93 7
	6	5½	91 7
	22	20½	93 2

smears thrice daily at 8 hour intervals When the infantile animal is given the total dose in one injection the period of induced oestrus is still shorter With this technique of administration much difficulty was experienced in arriving at a satisfactory evaluation of the response because of the persistence of leucocytes even at high dose levels

The short duration of the cornified period in the immature rat undoubtedly accounts for the inconsistent results obtained by Brownlee (reported by Burn (5)) who made but one observation of the vaginal contents 4 days after the first of six injections spread over 56 hours. In our experience cornification was encountered on the fourth or fifth day after the beginning of administration, the induction period is thus about 24 hours longer than in the anoestrous adult. By the recording of smears at least twice daily over the critical period regular responses have been obtained with the infantile animal by many investigators (Chapman (2), Hamburger and Pedersen Bjergaard (6), and others) and in the present instance.

TABLE 3

Parameters of the transformed dosage response curves with their standard errors, and accuracy of the methods

TEST	NUMBER OF ANIMALS	SLOPE	$\log_{10}$ O.D. 50	O.D. 50	LIMITS OF ERROR
		$b \pm s_b$	$m \pm s_m$	μ grams	per cent
Anoestrous adult rat	561	8.72 ± 1.03	0.525 ± 0.009	3.35	76–131
Infantile rat (divided dose)	244	5.45 ± 0.68	0.966 ± 0.016	9.24	65–154
Infantile rat (single injection)	210	3.14 ± 1.03	1.364 ± 0.047	23.1	47–212
Ovariectomized rat (oestrone)†	300	3.31 ± 0.46	0.114 ± 0.024	1.30	49–203

The limits of error are expressed as percentages of the true result within which the estimate will lie 99 out of every 100 times that the test is carried out (i.e. $P = 0.99$) and computed (Equation (10) of Gaddum (3)) in accordance with the recommendations of the Sub-Committee on the Accuracy of Biological Assays of the British Pharmacopoeia Commission (7). The calculations are made on the assumption that the standardization curves are used for the estimation of the potency of an unknown extract by the method of Gaddum (method (b) of the previous communication (1)) and that 20 animals receive the preparation being tested, and 20 the standard preparation, the responses in each case being 50 per cent.

† Included for comparative purposes. Data pertaining to the standardization of oestrone in ovariectomized rats of the same strain (single injection in oil, smears recorded thrice daily).

The extent of the group variation in sensitivity is approximately the same with both test objects. With the infantile animal (divided dose) two of the twenty-one observations lie beyond the limits of ±2 standard deviations (fig. 2), as against three out of twenty-seven for the adult animal (1, fig. 6).

A comparison of the parameters of the different regression lines (table 3) shows that the characteristic curve for the anoestrous adult is significantly steeper than that for the infantile animal by either method of administration. Hence the limits of error of the adult rat test are narrower. Whether this increase of accuracy is worth the additional labour entailed in the preparation of the test object is doubtful but it should be emphasized that a minimum of

forty females 21 days of age are required to confine the error of the infantile rat test to the limits stated In most laboratories the number available on any one day is considerably less

As would be expected, the dietary anoestrous adult rat *is much more sensitive* to gonadotrophic substance, only about one-seventh the quantity being required under the same conditions of administration to produce a 50 per cent response (table 3), per unit of body weight this ratio is approximately 1 to 21 There is no reason to suppose that the unripe follicles of the infantile ovary would require any stronger stimulus to secrete a given amount of oestrin than the immature follicles of the atretic ovary which remain unaffected by the dietary deficiency On the other hand, a greater and more prolonged output of the secondary hormone is certainly demanded by the infantile animal for the stimulation of the undeveloped vagina In the adult rat test the period of experimental anoestrus is hardly long enough to permit any appreciable atrophy of the secondary organs The greater degree of vaginal atrophy that follows castration undoubtedly accounts for the fact that, in the assay of oestrin in the ovariectomized adult, the rate of absorption of this hormone must be delayed by spreading the dose or use of an oily solvent in order to produce a cornified period of reasonable duration, whilst the administration of a single injection of the primary gonadotrophic hormone to the anoestrous adult is followed by a cornified period of 24 or more hours

The adult rat assay thus offers several important advantages over the infantile rat test

(*a*) Large numbers of test objects can readily be made available

(*b*) Age does not influence the reaction

(*c*) A single injection of gonadotrophic substance induces a period of cornification of 24 or more hours duration which necessitates smearing only once each day and greatly facilitates the evaluation of the response

(*d*) The method is more economical in that the same test objects can be used repeatedly

(*e*) The test object is more sensitive

(*f*) Individual variation in sensitivity is less (steeper dosage-response curve) and hence greater accuracy is achieved

The chief disadvantage of the method is that preparation must be carried out before the test

SUMMARY

1 Dose-effect curves have been constructed relating the induction of vaginal oestrus in the 21 day old rat and the dose of gonadotrophic substance administered (*a*) in a single injection and (*b*) over a period of 3 days

2 Subdivision of the dose is necessary with this test object in order to obtain uniform responses

3 The slope of the regression line for the infantile animal (divided dose)

is significantly flatter than that pertaining to the dietary anoestrous adult rat of the same strain. Hence the accuracy of the latter test is greater When twenty animals receive the standard preparation and twenty receive the preparation being tested and the response in each case is 50 per cent the limits of error at $P = 0\ 99$ are 65 and 154 per cent for the infantile rat test and 76 and 131 per cent for the adult rat test.

4 The advantages of the adult rat method are discussed

The skilful technical assistance of Mr G T Perry is gratefully acknowledged

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THE ELECTROLYTIC DISSOCIATION OF MORPHINE DERIVATIVES AND CERTAIN SYNTHETIC ANALGETIC COMPOUNDS

FRED W OBERST[1] AND HOWARD L ANDREWS[2]

From the U S Public Health Service Hospital, Lexington, Kentucky

Received for publication October 14, 1940

For several years a number of organizations[3] have been cooperating in an attempt to produce a substance which would be as useful clinically as morphine, but which would have little or no addiction-producing properties. During the course of this work many new drugs have been prepared The analgetic power of these drugs covers a wide range (1), some being weaker and some more powerful than morphine In some cases structural modification resulted in marked changes in analgetic or toxic actions It would be highly desirable to find some physico-chemical property which might be correlated with analgetic power and toxicity and which would serve as a guide toward the production of drugs more clinically useful It was thought that perhaps electrical conductivity measurements might be of value, since these measurements essentially determine the degree of dissociation and the ionic mobility, properties which might be of fundamental importance

Many of the alkaloidal bases are very insoluble in water, unstable, or difficult to prepare Consequently only five of the drugs were studied in the form of the free base, some as the base and the hydrochloride, and others only as the salt All of these drugs were prepared by Dr L F Small and collaborators The alkaloidal derivatives were purified to constant optical rotation and constant melting point and the synthetic compounds were of analytical purity

All measurements were made on solutions in conductivity water Any water having a specific resistance less than 1 5 megohm per cm was discarded All conductivity measurements were made in a Washburn type cell with platinized electrodes The cell was mounted in a water bath maintained at 25 ± 0 02°C The bridge was constructed of General Radio non-inductive decade resistors arranged in a Wheatstone bridge circuit with condensers

[1] Biological Chemist, U S Public Health Service

[2] Associate Physicist, U S Public Health Service

[3] The organizations taking part are the Rockefeller Foundation, the National Research Council, the United States Public Health Service, the United States Bureau of Narcotics, the University of Virginia, the University of Michigan, and the National Institute of Health

inserted for balancing the cell capacitance. The bridge was fed from a vacuum tube oscillator which furnished about 15 volts at 1000 cycles with a

TABLE 1

The equivalent conductance of certain alkaloidal salts and synthetic drugs

PREPARATION NUMBER	HYDROCHLORIDE SALTS OF ALKALOIDS AND SYNTHETICS	EQUIVALENT CONDUCTANCE	TOXICITY*	ANALGESIA
		Λ_0	M.L.D	M.E.D
128	Diacetyldihydromorphine	94 7	119	1 82
150	3-[2-(Diethylamino) 1 hydroxy-ethyl] phenanthrene	95 0		
265	Dihydro-α-isomorphine	96 1	890	0 80
30-B	Tetrahydro-α-methylmorphimethine	96 4		
3	Pseudocodeine	96 5	1780	17 8
1	Morphine	96 6	531	0 75
37	Dihydromorphine	96 9	183	0 26
2	Codeine	97 1	241	8 04
471	Tetrahydro-α-morphimethine	97 4	700	100 00
127	Diacetylmorphine (Heroin)	97 7	262	0 43
236	α-Isomorphine	97 8	890	0 80
256	3-Hydroxy-6-[2-(diethylamino) 1 acetoxy ethyl] phenanthrene	97 9		
409	2 [3 (Dimethylamino) 1 hydroxy propyl]-9-methyl carbazole	97 9		
359	Methyldihydromorphinone	98 0	25	0 07
149	3 [2 (Dimethylamino) 1 hydroxy ethyl] phenanthrene	98 3		
340	2 [2 (Dimethylamino) 1 hydroxy-ethyl] 9 10-dihydrophenanthrene	98 9		
342	2 [2 (Diethylamino) 1 hydroxy ethyl] 9 10-dihydrophenanthrene	99 1		
257	3 Dimethylamino 4 hydroxy 1 2 3 4 tetrahydrophenanthrene	99 3		
199-A	Monoacetylmorphine	100 7	293	0 18
322	Dihydrohydroxycodeinone (Eukodal)	101 0	426	1 34
87	Tetrahydrothebaine	101 9	117	1 16
352	3 [3 (Diethylamino) 1 hydroxy - propyl] phenanthrene	103 4		
346	2 [2 (Tetrahydroisoquinolino) 1 hydroxy ethyl] 9 10-dihydrophenanthrene	107 2		

Toxicity and analgesia as determined by Eddy (1) Values taken from Supplement no 138 to Public Health Reports 1938. Data on certain synthetic drugs have not been completed.

pure wave form Two stages of amplification were inserted between the bridge output and the telephone detector

In the case of the salts a plot of equivalent conductance (Λ_0) against the

square root of concentration ($\sqrt{c}$) yielded a straight line as predicted by Onsager (2) The values of Λ_0 are listed in table 1 All values of Λ_0 fell between the limits of 94 7 and 107 2

It has been reported that Heroin hydrochloride in cold aqueous solution hydrolyzes with a loss first of one and finally both acetyl groups after a period of several months (3) To determine the rapidity with which this hydrolysis takes place, a solution was placed in a conductivity cell and a balance made as rapidly as possible There was no appreciable change (0 1 per cent) in conductivity from one minute after the time of solution to two weeks after solution Hence we believe that hydrolysis must proceed at a very slow rate

The dissociation constants for morphine, dihydromorphine, codeine, dihydrocodeine and tetrahydro-α-morphimethine are given in Table 2

TABLE 2

Dissociation constants of certain alkaloidal bases as determined by conductivity methods

ALKALOIDS	$\frac{1}{\Lambda_0}$ (FREE BASE)	$\frac{1}{\Lambda_0}$ CALCULATED FROM THE HCl SALT	DISSOCIATION CONSTANT K	TOXICITY	ANALGESIA
				M L.D	M.E.D
Morphine	0 00433	0 00454	$1\ 64 \times 10^{-6}$	531	0 75
Dihydromorphine	0 00466	0 00452	$2\ 26 \times 10^{-6}$	133	0 26
Codeine	0 00442	0 00451	$1\ 61 \times 10^{-6}$	241	8 04
Dihydrocodeine	0 0143	No salt	$5\ 65 \times 10^{-6}$	225	7 20
Tetrahydro-α-morphimethine	0 00467	0 00455	$4\ 50 \times 10^{-6}$	700	100 0

* The $\frac{1}{\Lambda_0}$ for the base was also calculated from conductivity data of the salt using values of Λ_0 for Cl^- as 76 34 and for OH^- as 198 (MacInnes page 342) (7)

From the calculated values of K it is noted that the hydrogenated morphine and codeine bases are stronger than their respective unhydrogenated compounds

Comparatively little work has been done on the electrical properties of the opium alkaloids Kolthoff (4), from solubility measurements, found the dissociation constant for morphine to be $1\ 35 \times 10^{-6}$ Baggesgaard-Rasmussen and Reimers (5), using a similar method, reported a value of $9\ 9 \times 10^{-7}$ for morphine Our value of $1\ 64 \times 10^{-6}$ is slightly greater than the previously reported figures

Our value of K for codeine, $1\ 61 \times 10^{-6}$, is larger than that given by Kolthoff (9×10^{-7}) or by Baggesgaard-Rasmussen and Martin (6) ($7\ 9 \times 10^{-7}$) The comparatively high solubility of codeine permitted measurements to be made over a wide range of concentrations, and as a result we feel that our

value for codeine is at least as reliable as the values reported by the other workers. No values for the dissociation constant of dihydromorphine dihydrocodeine, and tetrahydro-α-morphimethine have been found in the literature.

All of the drugs reported here have been studied in normal animals and many in human drug addicts Some information is available as to their analgetic power and toxicity (1) As far as can be determined, there is no correlation between these properties of the drugs and the value of either K or Λ_0 (table 2)

CONCLUSIONS

As far as can be determined, there is no correlation between analgetic or toxic properties of the drugs and the values of either the dissociation constants or equivalent conductance at infinite dilution. There is no evidence that an aqueous solution of diacetylmorphine hydrochloride (Heroin) is unstable from one minute to two weeks after its preparation The hydrogenated morphine and codeine are stronger bases than their respective unhydrogenated compounds

The authors wish to thank Dr Erich Mosettig who suggested the problem, and Dr L. F Small for his interest throughout the work.

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THE EFFECTS OF CERTAIN CHEMICAL CHANGES ON THE ADDICTION CHARACTERISTICS OF DRUGS OF THE MORPHINE, CODEINE SERIES

C K HIMMELSBACH[1]

From the United States Public Health Service Hospital, Lexington, Kentucky

Received for publication October 15, 1940

The results of studies of the relation of changes in chemical structure to alterations in certain physiological effects of drugs of the morphine-codeine series were summarized by Small and Eddy in 1938 (1, Part I) They found that (a) methylation of the phenolic OH of morphine increased its convulsant action and sometimes its toxicity, but decreased other effects, (b) replacement of the alcoholic OH with H or O increased but usually shortened the effects, (c) spatial shift of the alcoholic OH generally caused increased effects, and, (d) saturation of the C-7, 8 double bond produced irregular effects This report presents the results of clinical studies on the effects of these types of chemical modification on two addiction characteristics, namely, *duration* and *potency* of physical dependence action

METHODS

Dihydromorphine (H-2-M), alpha-isomorphine (iso-M), dihydro-alpha-isomorphine (H-2-iso-M), dihydromorphinone (Dilaudid), dihydrodesoxymorphine-D (deso-M), codeine (C), dihydrocodeine (H-2-C), isocodeine (iso-C), dihydroisocodeine (H-2-iso-C) and dihydrodesoxycodeine D (deso-C) were each studied by the substitution technique described in previous reports (1, Part III, and 2) Briefly, this technique involves (a) selection of addicts with active physical dependence, (b) preliminary stabilization of patients on morphine, (c) replacement of morphine by the substance in question for a period of at least one week, and, (d) withdrawal of the substituted drug Thus it can be learned whether or not a given substance will satisfy pre-established physical dependence The maintenance of physical dependence by a substituted drug is considered to indicate that it is capable of producing addiction The majority of the morphine and codeine derivatives which are known to have caused addiction have been tested by substitution, and all satisfied pre-established physical dependence No non addictive drug has been found to support physical dependence

The physical dependence already established to morphine apparently becomes adapated to the substitute, for, upon withdrawal, detectable and self-consistent dissimilarities have been found in the abstinence syndromes of drugs studied These dissimilarities, thought to be due to differences in physical dependence action, are based on alterations in chemical structure The abstinence syndrome which appears following withdrawal of the substituted drug is thought to be the equivalent of that which

[1] Passed Assistant Surgeon, U S Public Health Service

would occur were addiction to the substituted drug primary instead of secondary Any morphine which may have been stored prior to substitution is thought to be excreted or destroyed

The chief differences encountered in drugs of the morphine, codeine series have been in potency and duration of physical dependence action. The methods used to obtain values expressing potency and duration of physical dependence action were described in a previous report (2) In brief values for potency are obtained by comparing the mean amounts of single equally effective physical dependence satisfying doses of morphine and of the substitutes In order to standardize the results these values are corrected to be equivalent to an arbitrary dose (60 mgm.) of morphine sulfate. Since potency varies inversely with the dose the corrected values are expressed as their reciprocals. Values considered to express satisfactorily the duration of physical dependence action are obtained by scoring the abstinence syndrome intensity (A.S.I) at hourly intervals following the last dose and using the time at which the group mean A.S.I has reached fifty per cent of its maximum.

RESULTS

Reports have been made on the results of substitution studies of H 2-M, deso-M deso-C (2), of C (3) and of Dilaudid (4) The pertinent data on these drugs, together with those reported for the first time in this paper are summarized in table 1 Data presented in table 2 permit comparison of certain factors concerned in physical equilibrium of patients before and after substitution of H 2-C iso-C H 2 iso-C, iso-M and H 2-iso-M Graphs of the daily group mean A.S I during morphine stabilization, substitution and following withdrawal of the latter drugs are presented in figure 1, and graphs of the onsets of the abstinence syndromes of the above drugs and of Dilaudid are shown in figure 2

Dihydrocodeine[1] Physical dependence was satisfied by this drug. Several patients complained that it caused itching and unpleasant dreams. The abstinence syndrome which followed its withdrawal was definitely less severe than that of morphine. The potency of dihydrocodeine was calculated to be $\frac{1}{15}$ and the duration of its physical dependence action was twenty hours

Isocodeine[1] Satisfaction of physical dependence by this drug was prompt and adequate The abstinence syndrome which followed its withdrawal was definitely less severe than that of morphine. The potency of isocodeine was calculated to be $\frac{1}{10}$ and the duration of its physical dependence action was eighteen hours

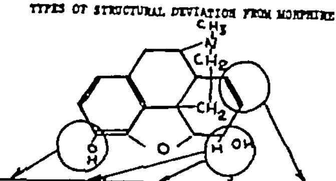
Dihydroisocodeine acid tartrate Satisfaction of physical dependence by this drug was neither prompt nor complete even though doses were administered

[1] Dihydrocodeine and isocodeine were furnished as alkaloidal bases Weighed amounts of the bases were dissolved in 1 N HCl neutralized to pH 7 and diluted to a convenient concentration prior to administration The values for potencies of these drugs are given in terms of their respective acid tartrates The acid tartrate of H 2-C known as 'Paracodin contains 64 3 per cent base and that of iso-C contains 66 6 per cent base

five times daily in amounts averaging four times those of morphine Nevertheless, the abstinence syndrome was nearly as severe as that which follows withdrawal of morphine The potency of physical dependence action of

TABLE 1

TYPES OF STRUCTURAL DEVIATION FROM MORPHINE



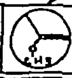
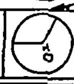
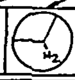
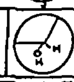
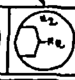
Drugs	Physical Dependence Action: Potency	Physical Dependence Action: Duration					
M	1/50	14					
H 2-M	1/15	19					x
iso-M	1/37	13				x	
H-2-iso-M	1/27	16				x	x
Dilaudid	1/7	7		x			x
deso-M	1/10	4 5			x		x
C	1/259	16	x				
H-2-C	1/276	20	x				x
iso-C	1/270	18	x			x	
H-2 iso-C	1/194	13	x			x	x
deso-C	1/70	6 5	x		x		x

TABLE 2

Comparison of several measures of physiologic equilibrium and dosage during morphine stabilization and after substitution of five derivatives

	M to H 2-C		M to iso-C		M to H-2-iso-C		M to iso-M		M to H 2-iso-M	
Temperature rectal, °C	37 1	37 0	37 1	37 1	37 0	37 1	37 1	37 1	37 0	37 0
Respiratory rate	15	15	17	16	15	16	16	16	16	16
A M Systolic B P	108	106	108	108	110	114	110	110	112	110
Caloric intake	3140	2940	3240	3240	3050	2690	3160	3150	2630	2500
A.M weight (last day)	69 10	69 36	73 50	74 09	66 11	65 69	68 57	69 41	57 49	57 60
Total daily dose (mgm)	135	475	166	666	271	1322	230	184	204	73
Number of daily injections	4	4	4	4	4	5	4	4	4	4
Number of patients	8		7		8		10		11	

dihydroisocodeine was calculated to be $\frac{1}{194}$ and the duration of this effect was thirteen hours

Alpha-isomorphine-hydrochloride Satisfaction of physical dependence upon substitution of this drug was prompt and adequate Its potency was calculated to be $\frac{1}{37}$ The abstinence syndrome which followed its withdrawal

was about as intense as that of morphine Analysis of the data on the onset of the abstinence syndrome showed the duration of physical dependence action of this drug to be thirteen hours.

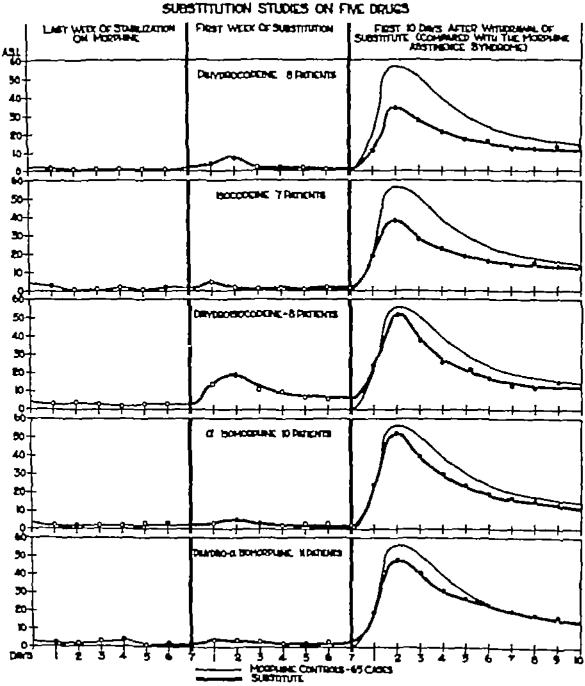


FIG 1

Dihydro-alpha isomorphine hydrochloride Satisfaction of physical dependence by this drug was prompt and adequate Its potency was calculated to be $\frac{1}{37}$ The intensity of the abstinence syndrome which followed its

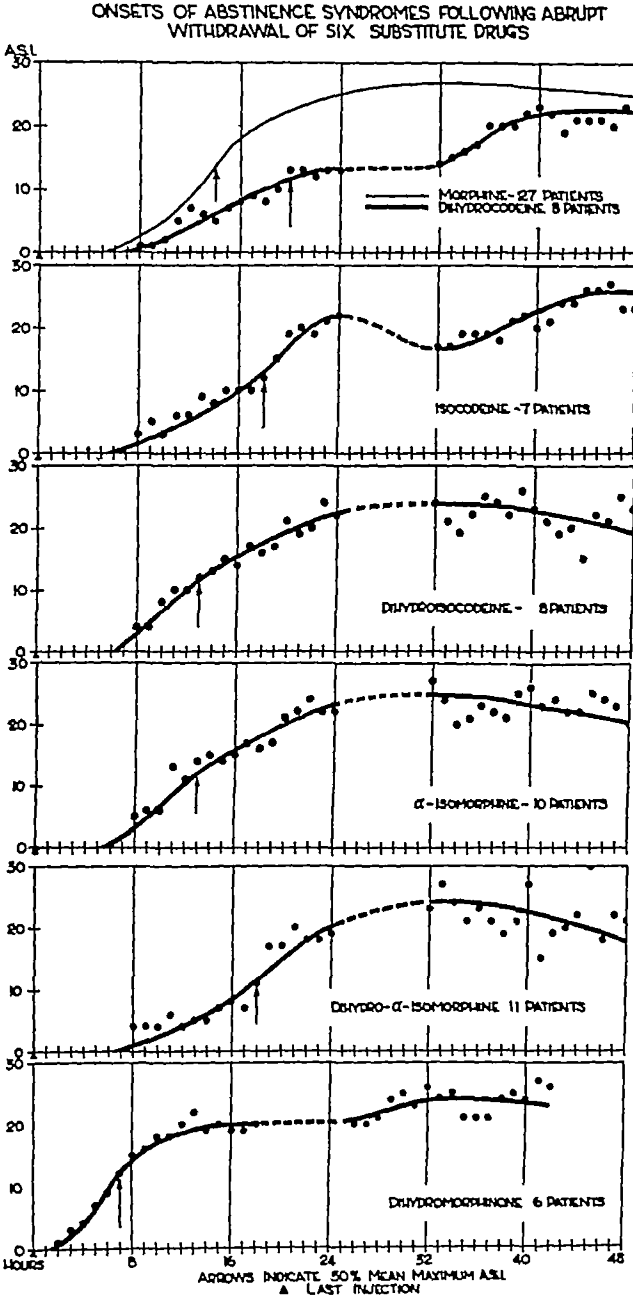

ONSETS OF ABSTINENCE SYNDROMES FOLLOWING ABRUPT WITHDRAWAL OF SIX SUBSTITUTE DRUGS
A.S.I.
30
20
10
0
MORPHINE - 27 PATIENTS
DIHYDROCODEINE 8 PATIENTS
ISOCODEINE - 7 PATIENTS
DIHYDROISOCODEINE - 8 PATIENTS
α-ISOMORPHINE - 10 PATIENTS
DIHYDRO-α-ISOMORPHINE 11 PATIENTS
DIHYDROMORPHINONE 6 PATIENTS
HOURS
8
16
24
32
40
48
ARROWS INDICATE 50% MEAN MAXIMUM A.S.I.
▲ LAST INJECTION

FIG 2

withdrawal was about as severe as that which follows withdrawal of morphine. The duration of its physical dependence action was eighteen hours.

Dihydromorphinone hydrochloride (Dilaudid) This drug was administered to a group of five morphine addicts merely for the purpose of obtaining data on the duration and potency of its physical dependence action. Owing to the brevity of its physical dependence action (seven hours) it was administered six times daily Doses one seventh as large as morphine satisfied physical dependence on this schedule, whereas doses one fourth as great as morphine

TABLE 3

The effects of altered structure on physical dependence action

	POTENCY	DURATION
(a) Methylation of the phenolic OH		
M to C	−	+
H 2 M to H 2-C	−	+
Iso-M to Iso-C	−	+
H 2-Iso-M to H 2-Iso-C	−	−
Deso-M to Deso-C	−	+
(b) Replacement of alcoholic OH by H or O		
H 2-M to Deso-M Dilaudid	+ +	− −
H 2-Iso-M to Deso-M, Dilaudid	+ +	− −
H 2-C to Deso-C	+	−
H 2-Iso-C to Deso-C	+	−
(c) Spatial shift of the alcoholic OH		
M to Iso-M	+	−
H 2-M to H 2 Iso-M	−	−
C to Iso-C	−	+
H-2-C to H 2 Iso-C	+	−
(d) Saturation of C-7 8 double bond		
M to H 2-M	+	+
Iso-M to H 2-Iso-M	+	+
C to H 2-C	−	+
Iso-C to H 2-Iso-C	+	−

were administered four times daily in a previous study (4 and 1 Part III)

Data on the duration and potency of physical dependence action of morphine, codeine, and nine of their derivatives make possible nineteen paired comparisons in which one drug of each pair differs from the other in only one structural respect. Such comparisons presented in table 3 yield information pertaining to the influence of four types of structural alteration on these addiction characteristics.

SUMMARY

By means of the substitution technique, data were obtained on the potency and duration of physical dependence action of each of ten related drugs of the morphine, codeine series From comparisons of pairs of drugs, differing from each other in only one structural respect, it would appear that in so far as physical dependence action is concerned

(a) Methylation of the phenolic OH reduces potency and prolongs action,
(b) Replacement of the alcoholic OH by H or O increases potency and shortens the action,
(c) Spatial shift of the alcoholic OH results in irregular effects, and
(d) Saturation of the C-7, 8 double-bond tends to increase both the potency and duration of action

These findings are qualitatively similar to those of Small and Eddy (1, Part I) in so far as the effects of (a) methylation of the phenolic OH and (b) replacement of the alcoholic OH by H or O are concerned, but are qualitatively somewhat dissimilar to their findings on the effects of (c) spatial shift of the alcoholic OH and (d) saturation of C-7, 8 double bond The dissimilarities can be considered favorable to the assumption of a dissociation of physical dependence action from other morphine effects None of the types of chemical modification mentioned in this report caused reductions in both duration and intensity of physical dependence action

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A COMPARISON OF THE RESPONSE OF YAWS AND SYPHILIS IN THE RABBIT TO THERAPY WITH MAPHARSEN AND NEOARSPHENAMINE

B J LONGLEY N M. CLAUSEN AND A L TATUM

From the Department of Pharmacology of the University of Wisconsin, Madison

Received for publication November 1 1940

It is the consensus of most investigators that clinical yaws is as difficult to cure as is syphilis (1), yet on the experimental side it appears that adequate therapeutic studies are wanting Nichols (2) did make an interesting comparison of the two experimental diseases, though on the basis of more modern techniques his results must be considered to be inadequate since his only criteria of cure were the regression of lesions and the disappearance of positive serological reactions. Neither lymph node nor tissue transfers were made to determine sterilization, hence conclusive results were not obtained

While a considerable amount of work (2–8) has been done on the comparative pathogenicity and immunology of yaws and syphilis in rabbits and in monkeys, there is a glaring paucity of information on the subject of therapy It is, therefore the object of this paper to fill in this gap so far as present facilities permit. Information on this phase of the subject might possibly have some bearing on the academic question of identity of the two "diseases," providing the responses to therapy were significantly different.

The Nichols strain of *Treponema pallidum* maintained in this laboratory since 1923 was used in these studies and the yaws strain was one generously supplied by Dr Alan M Chesney The two diseases were maintained by testicular transfer in the same laboratory under as nearly similar conditions as possible Rabbits with typical lesions were selected for the therapeutic studies and each animal was treated with a single intravenous injection of an arsenical drug Seven days later the testicular lesion was removed minced and extracted in 0 9 per cent sodium chloride solution. The extract so obtained was injected intratesticularly into two recipient rabbits which were then observed for from eight to twelve weeks for the appearance of lesions and positive blood Wassermann reactions. When the recipient animals failed to show lesions at the site of injection or a positive blood Wassermann reaction the original rabbit from which the transfer was made was considered cured These criteria of cure were selected since it has been observed that the results of lymph node transfers used so frequently in studies of rabbit syphilis, were irregular in rabbit yaws (4 7 9)

The results of mapharsen therapy in rabbit yaws and rabbit syphilis are given in table 1 It is evident that there is no significant difference in the

TABLE 1

Mapharsen therapy of rabbit yaws and rabbit syphilis using infectivity of testicular material as the criterion of cure*

DISEASE	DOSE OF MAPHARSEN† (MGM PER KGM OF BODY WEIGHT)	NUMBER OF RABBITS	NUMBER CURED	NUMBER NOT CURED
Yaws	2 0	5	1	4
Syphilis	2 0	4	2	2
Yaws	3 5	3	1	2
Syphilis	3 5	3	1	2
Yaws	5 0	3	3	0
Syphilis	5 0	3	2	1
Syphilis	7 0	2	2	0

* The testicle showing the largest lesion was selected for transfer which was made seven days after the administration of the drug

† Single dose administered intravenously

TABLE 2

Neoarsphenamine therapy of rabbit yaws and rabbit syphilis using infectivity of testicular material as the criterion of cure*

DISEASE	DOSE OF NEOARSPHENAMINE† (MGM PER KGM. OF BODY WEIGHT)	NUMBER OF RABBITS	NUMBER CURED	NUMBER NOT CURED
Yaws	4	1	1	0
Yaws	7	4	3	1
Syphilis	7	3	1	2
Yaws	13	3	3	0
Syphilis	13	3	3	0
Yaws	20	3	3	0
Syphilis	20	3	3	0
Yaws	50	5	5	0
Syphilis	50	1	1	0
Yaws	100	1	1	0

* The testicle showing the largest lesion was selected for transfer which was made seven days after the administration of the drug

† Single dose administered intravenously

response of the two diseases to therapy with this arsenical, nor is there a significant difference in the response of the two diseases to therapy with neoarsphenamine, as indicated in table 2. The fact that the two experimental diseases are cured with about equal ease is in agreement with the clinical results of Pardo-Castello (1) The writers recognize that equal susceptibility to cure has no necessary bearing on the identity of the two diseases. Rabbits infected with *Trypanosoma equiperdum* are cured by certain arsenical drugs in about the same dosage as required for rabbits infected with *Treponema pallidum*, yet no one would conclude that the two diseases are identical. It may be noted that yaws and syphilis are cured with equal ease, yet they can usually be differentiated with readiness on the basis of physical signs, as claimed by Turner and Chesney (5)

The foregoing data, using testicular transfer as the criterion of cure, indicate that the therapeutic index of single doses of neoarsphenamine is superior to that of single doses of mapharsen in the treatment of rabbit yaws and rabbit syphilis The rabbit tolerates 150 mgm. per kilogram of neoarsphenamine and mapharsen is tolerated at only 10 mgm. per kilogram whereas the single minimal curative doses are 13 and 5, respectively

These data should be read in the light of previously published evidence that multiple doses of mapharsen (10) are considerably more efficient than single doses whereas, according to Kolmer (11) neoarsphenamine is relatively less efficient. Consequently the significance of this contribution resides in its bearing on relative curability of yaws and of syphilis and not on the determination of conditions for the development of maximal therapeutic efficiencies.

SUMMARY AND CONCLUSIONS

The comparative response of rabbit yaws and rabbit syphilis to therapy with single doses of mapharsen and neoarsphenamine has been determined, and from the data obtained it appears that there is no essential difference in the ease with which the two diseases may be cured

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CHEMOTHERAPEUTIC ACTIVITY OF N[4]-ACYLSULFANILHYDROXAMIDES

BETTYLEE HAMPIL, G W WEBSTER, AND MAURICE L MOORE

From the Medical-Research Division, Sharp and Dohme, Inc, Glenolden, Pa

Received for publication October 10, 1940

Certain derivatives and analogues of sulfanilamide which possess varying degrees of efficacy in the treatment of bacterial infections have been synthesized and investigated Among them, it has been found (1, 2) that certain of the N^4-acyl derivatives of sulfanilamide possess low toxicity and are effective in combating experimental streptococcic infections in mice Following this, fourteen members of a series of N[4]-acylsulfanilhydroxamides have been synthesized (3) Inasmuch as several of these compounds, particularly those in which the acyl group contains four or more carbon atoms, are effective antistreptococcic agents of low toxicity, it has seemed of interest to report the chemotherapeutic data on the series [1]

Technic For Streptococcal Tests White mice, weighing 18–22 grams, were employed as test animals throughout the investigation These mice were of a standard stock strain, secured from one source

β-Hemolytic Streptococcus (Strain No 1685) has been used for routine chemotherapeutic tests for the detection of compounds possessing antistreptococcic activity This strain was isolated from a case of erysipelas and because of its broad antigenicity and high virulence has been employed since 1929 in the production of erysipelas antiserum.

The procedure adopted in growing and maintaining the virulence of the culture is that which has been employed for many years in the standardization of antistreptococcic serums If a culture is used every week it is passed once through mice before use If more than one week elapses between tests at least two and sometimes three mouse passages are executed in order to assure satisfactory virulence, which is determined by protection tests with a standard serum in conjunction with plate counts

The following routine for the preparation of the test cultures is carried out Mice are injected intraperitoneally with a 7-hour rabbit blood broth culture diluted 2×10^{-4} Eighteen to twenty hours later moribund animals are sacrificed and approximately 0 1 cc of heart's blood is planted into rabbit blood broth Blood agar plates are streaked for purity at the same time The following morning 0 2 cc of the culture is transferred to a tube containing 5 cc of warm Hygienic Laboratory broth[2] containing 10 per cent rabbit serum Five to six hours later, the required turbidity of 2×10^9 organisms per cc is reached Dilutions for the test are made with Dunham's peptone

[1] The chemotherapeutic activity of three members of the series, n-valeryl, n hexanoyl, and n-heptanoyl sulfanilhydroxamide, has been reported by Cooper, Gross and Lewis, Proc Soc Expt Biol Med, 43, 491, 1940

[2] Veal infusion broth containing 2 per cent neopeptone and 0 5 per cent NaCl adjusted to pH 7 4

water It has been found that when five or more mice are each injected intraperitoneally with 0.5 cc of the 5×10^{-8} dilution at least half of them succumb to the infection within 72 hours This dilution has been designated as the minimal lethal dose, and contains one to five organisms.

The treated animals were infected intraperitoneally with 1000 lethal doses of the streptococci A minimum of ten mice, not receiving treatment, were also infected with a similar dose five at the beginning and five at the end of each experiment. At the time of each test the culture was titrated for the determination of the minimal lethal dose thus assuring an infective dose of the required standard. Blood agar plates were made with 0.5 cc. of the three highest dilutions for estimation of the number of viable organisms per minimal lethal dose.

TABLE 1

N^4-Acylsulfanilhydroxamides

Infection Streptococcus (strain #1685) 1000 lethal doses.

Treatment Single dose administered orally simultaneously with injection of the culture

COMPOUNDS	DOSE RANGE MGM. PER MOUSE*	NUMBER OF TESTS	NUMBER OF MICE	PER CENT OF ANIMALS SURVIVING† DAYS				
				1	2	3	4	5
Controls		8	106	51	5	2	2	0
Sulfanilamide	12–60‡	7	104	93	91	85	75	69
N^4-Propionylsulfanilhydroxamide	12–200	1	27	85	48	26	4	0
N^4-Butyrylsulfanilhydroxamide	12–200	3	69	100	93	87	68	65
N^4-Valerylsulfanilhydroxamide	12–200	3	68	100	100	98	88	78
N^4-Hexanoylsulfanilhydroxamide	12–200	4	87	96	94	79	71	63
N^4-Heptanoylsulfanilhydroxamide	12–200	2	48	98	96	85	73	73
N^4-Octanoylsulfanilhydroxamide	12–60§	1	12	100	100	92	83	83
N^4-Nonanoylsulfanilhydroxamide	12–200	1	24	100	75	33	12	3
N^4-Isovalerylsulfanilhydroxamide	12–200	1	24	92	75	54	33	25
N^4-Isohexanoylsulfanilhydroxamide	12–150	1	20	100	100	100	95	90

* The compounds were administered to groups of mice at 12, 24 48 60 80 100, 150 and 200 mgm. doses.

† The animals treated with each compound were observed individually and the results pooled for calculation of the percentage survival for each day

‡ Larger doses not given because of acute toxicity

§ Larger doses not given because of inability to administer the compound in more concentrated suspensions.

The chemical compounds were suspended in 0.5% tragacanth and administered orally in a volume of 0.5 cc

The mice were observed daily for five to seven days, as recorded in the tables.

RESULTS

1 *Anti streptococcal data*

a Single dose treatment. In the earlier experiments, a single dose of each chemical compound was given simultaneously with the infective organisms at dose levels ranging from 12 to 200 mgm. per mouse (table 1) The adminis-

tration of the compounds in this manner served a two-fold purpose, since a preliminary indication of both the chemotherapeutic activity and the relative toxicity of the compounds is obtained Doses of more than 60 mgm. of sulfanilamide were not employed because of the acute toxicity of this drug in this and larger amounts The unacylated compound, sulfanilhydroxamide,[3] is also toxic in doses of more than 60 mgm On the other hand, the acyl derivatives of sulfanilhydroxamide appear to be innocuous in a single dose of 200 mgm

Examination of the data in table 1 indicates that five members of the series, *n*-butyryl, *n*-valeryl, *n*-hexanoyl, *n*-heptanoyl and *n*-octanoyl, possess promising antistreptococcic properties

b Four dose-four day treatment As the work progressed a dosage schedule was developed which permitted a more rigid comparison of the chemotherapeutic activity of each compound with that of sulfanilamide than was afforded by the previous tests

The results obtained from a series of exploratory tests, using 1 5 to 15 mgm doses, indicated that four doses of 5 mgm each administered at 24-hour intervals provided a suitable treatment schedule for the compounds, the first dose was administered simultaneously with the culture In our hands, this treatment has proved satisfactory for purposes of general comparison with sulfanilamide of these, and other compounds of similar solubility In 38 separate tests, made over a period of two years and employing a total of 600 mice, we have found that 20 to 25 per cent of the animals thus treated with sulfanilamide survive for a selected observation period of seven days This figure (20 to 25 per cent) allows a more critical evaluation for comparative purposes than could be obtained with a greater survival percentage

When sulfapyridine became available it was also included on each test and gave results almost identical with those of sulfanilamide

The number of tests made on each compound, together with the results obtained, are recorded in table 2 A minimum of 10 mice was used in each test on a single compound, several tests employed as many as 50 mice It may be observed from the data that here again *n*-valeryl, *n*-hexanoyl, and *n*-heptanoyl are the most active derivatives of the group It would appear that these three compounds compare favorably with sulfanilamide and sulfapyridine under the conditions of this test

In a single set of experiments the three most active members of the acylsulfanilhydroxamides, together with sulfanilhydroxamide, sulfanilamide and sulfapyridine, were tested at an equivalent dose level calculated in terms of millimoles per mouse, rather than milligrams The results are given in table 3 and indicate, as was anticipated, that mole for mole, the *n*-valeryl,

[3] The synthesis of sulfanilhydroxamide and acetylsulfanilhydroxamide has been reported by Kharasch and Reinmuth, U S Patent 2,097,414, Chemical Abstracts, **32**, 1407, 1938

TABLE 2

N^4-Acylsulfanilhydroxamides

Infection. Streptococcus (strain #1685) 1000 lethal doses.

Treatment Four 5-mgm doses orally at 24-hour intervals first dose administered simultaneously with injection of the culture

COMPOUNDS	DAILY DOSE MGM. PER MOUSE	NUMBER OF TESTS	NUMBER OF MICE	PER CENT OF ANIMALS SURVIVING DAYS						
				1	2	3	4	5	6	7
Controls	5	21	225	20	2	1	1	0		
Sulfanilamide	5	16	491	100	81	68	63	50	35	23
Sulfapyridine	5	5	150	99	91	63	58	43	29	23
Sulfanilhydroxamide	5	2	25	96	48	28	20	20	20	8
N^4-Acetylsulfanilhydroxamide	5	2	25	85	20	5	5	5	5	5
N^4-Valerylsulfanilhydroxamide	5	5	155	100	95	88	83	63	50	38
N^4 Hexanoylsulfanilhydroxamide	5	23	605	100	90	82	79	73	52	36
N^4-Heptanoylsulfanilhydroxamide	5	6	171	100	96	86	81	70	53	37
N^4-Isobutyrylsulfanilhydroxamide	5	1	15	87	27	7	7	0		
N^4-Succinylsulfanilhydroxamide	5	1	20	55	5	0				
N^4-Maleylsulfanilhydroxamide	5	1	10	60	0					
N^4-Benzoylsulfanilhydroxamide	5	1	10	90	20	0				
p-Nitrobenzenesulfonhydroxamide	5	1	10	100	100	50	30	30	30	30
p-Toluenesulfonhydroxamide	5	1	10	10	10	10	10	10	10	10
Benzenesulfonhydroxamide*	5	1	10	100	0					
Benzhydroxamic Acid	5	1	10	100	10	0				

* These compounds are not members of this series, but are included for purposes of comparison

TABLE 3

Comparison of chemotherapeutic activity of certain acylsulfanilhydroxamides sulfanilamide and sulfapyridine on the basis of molar equivalent doses

Infection Streptococcus (strain #1685) 1000 lethal doses.

Treatment Four 0.03 millimole doses orally at 24-hour intervals first dose administered simultaneously with injection of the culture.

COMPOUNDS	DAILY DOSE, MILLIMOLES PER MOUSE	MGM. PER DOSE	NUMBER OF TESTS	NUMBER OF MICE	PER CENT OF ANIMALS SURVIVING DAYS						
					1	2	3	4	5	6	7
Controls			1	10	10	0					
Sulfanilamide	0.03	5.16	1	15	100	60	40	33	26	13	0
Sulfapyridine	0.03	7.47	1	15	100	87	47	40	33	20	7
Sulfanilhydroxamide	0.03	5.64	1	15	100	93	47	33	7	7	0
N^4 Acetylsulfanilhydroxamide	0.03	6.90	1	15	87	13	0				
N^4-Valerylsulfanilhydroxamide	0.03	8.16	1	15	100	100	100	100	73	40	40
N^4-Hexanoylsulfanilhydroxamide	0.03	8.58	1	15	100	100	100	93	87	53	26
N^4-Heptanoylsulfanilhydroxamide	0.03	9.00	1	15	100	100	100	100	100	60	40

n-hexanoyl and n-heptanoyl derivatives exceed sulfanilhydroxamide, sulfanilamide, and sulfapyridine in chemotherapeutic efficiency against experimental streptococcal infections in mice under these experimental conditions

2 Anti-meningococcal data

A series of tests has also demonstrated the activity of the compounds against the meningococcus (table 4) In these tests experimental infections in Swiss mice were produced with a strain of Type I meningococcus by the use of mucin, according to the method of Miller and Castles (4) The treatment of the animals varied from that used in the antistreptococcic tests in that the dose was increased from 5 to 20 mgm, and an extra dose was given seven hours after the infection

TABLE 4

Results with meningococcus Type I, 100 to 1000 lethal doses

Treatment Five 20 mgm doses orally First dose administered simultaneously with injection of the culture, and at intervals of 7, 24, 48 and 72 hours

COMPOUND	SINGLE DOSE, MGM PER MOUSE	NUMBER OF TESTS	NUMBER OF MICE	PER CENT OF ANIMALS SURVIVING DAYS						
				1	2	3	4	5	6	7
Controls		3	25	52	8	4	4	4	4	4
Sulfanilamide	20	3	30	93	63	43	30	23	23	23
Sulfapyridine	20	3	30	76	50	50	30	23	20	20
N^4-Valerylsulfanilhydroxamide	20	2	20	85	35	35	35	30	30	30
N^4-Hexanoylsulfanilhydroxamide	20	3	30	90	47	40	26	23	20	20
N^4-Heptanoylsulfanilhydroxamide	20	2	20	95	40	20	15	15	15	15

3 Blood studies

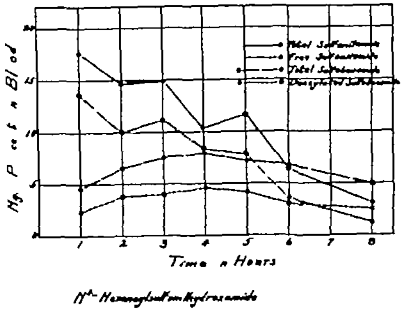
In order to correlate the chemotherapeutic effectiveness of these compounds with the blood concentrations attained we have determined the blood levels produced in mice on the dosage schedules employed in the above experiments In this report, N^4-hexanoylsulfanilhydroxamide has been selected as representative of the three more active compounds for comparison with sulfanilamide with respect to relative absorption and blood concentration on the five milligram dose level, and with sulfapyridine on the twenty milligram dose level

Sulfanilamide and sulfapyridine determinations were made by the methods of Marshall (5) N^4-Hexanoylsulfanilhydroxamide was determined by the following method [4]

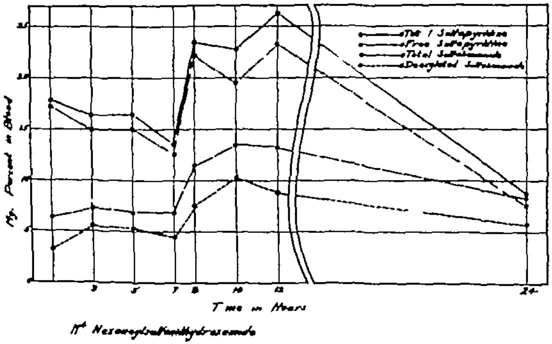
Add 1 cc of blood to 4 cc of saponin solution (0 5 gram per liter), after laking of blood (5 minutes) add 15 cc of 95 per cent ethanol and then 1 drop of p toluenesulfonic

[4] This is a modification of a method developed by Dr Hansen, Jefferson Medical College, which will be reported in complete detail elsewhere

acid solution (20 per cent) and allow to stand for 5 minutes before filtering. Place 5 cc. of the alcoholic blood filtrate in a 10 cc calibrated tube and evaporate on a water bath



GRAPH 1. COMPARISON OF THE BLOOD CONCENTRATION OF SULFANILAMIDE AND SULFABENAMIDE IN MICE AFTER A SINGLE 5 MGM DOSE ADMINISTERED ORALLY



GRAPH 2. COMPARISON OF THE BLOOD CONCENTRATION OF SULFAPYRIDINE AND SULFABENAMIDE IN MICE AFTER THE ORAL ADMINISTRATION OF TWO 20 MGM DOSES, WITH THE SECOND DOSE GIVEN ON THE SEVENTH HOUR

to 1 cc Add 2 cc. of p-toluenesulfonic acid to the solution, make up to 10 cc. volume with distilled water and heat on a water bath for 90 minutes. Cool adjust to 10 cc.

volume and filter To 5 cc of this filtrate add 0 5 cc of 0 1 per cent sodium nitrite and mix. After 3 minutes add 0 5 cc of 0 5 per cent ammonium sulfamate in 1 M sodium dihydrogen phosphate solution and mix After 3 minutes add 0.5 cc of 0 1 per cent N-(1-naphthyl) ethylene diamine dihydrochloride solution and mix After 10 minutes read the solution in the Klett colorimeter using filter #54. This gives the total N^4-hexanoylsulfanilhydroxamide, while the free or deacylated product is determined by evaporating 5 cc of the original alcoholic blood filtrate to 1 cc Cool and add 2 cc of p-toluenesulfonic acid and make up to 10 cc volume with distilled water Filter and use 5 cc of filtrate for diazotization as described above

The results of the blood tests are shown in the graphs Each determination was made from the pooled blood of five mice The data on the five milligram dose are shown on graph 1 and indicate that N^4-hexanoylsulfanilhydroxamide is absorbed more slowly than sulfanilamide and its maximum concentration is lower It also appears that over an eight hour period the concentration of the sulfonhydroxamide is maintained more uniformly The data on the 20 milligram dose as shown on graph 2 indicate that the same relationship holds true in comparison with sulfapyridine, in that the drug is absorbed more slowly and its concentration in the blood is lower

SUMMARY

Fourteen members of a series of N^4-acylsulfanilhydroxamides and four related compounds have been tested for chemotherapeutic activity Three of the derivatives, n-valeryl, n-hexanoyl and n-heptanoyl, possess a high degree of efficacy in combating experimental streptococcal infections in mice They have also been found to be effective against a strain of meningococcus

Blood concentrations of N^4-hexanoylsulfanilhydroxamide, sulfanilamide and sulfapyridine have been determined in mice and correlated with chemotherapeutic activity

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THE LOCAL ANESTHETIC ACTIVITY AND TOXICITY OF ALKAMINE ESTERS OF p-FLUOROBENZOIC ACID

E. E. CAMPAIGNE, A. C STARKE, JR. L. S FOSDICK AND C A. DRAGSTEDT

From the Department of Chemistry (Dental School) and the Department of Physiology and Pharmacology (Medical School) Northwestern University Chicago

Received for publication October 14, 1940

Recent investigations have demonstrated that fluorine can sometimes replace the hydroxyl group in compounds having physiological activity without the loss of that activity The remarkable inertness of organic fluorine (1) led two of the authors (2) to prepare some analogues of procaine containing fluorine in the *para* position. The following compounds (as hydrochlorides) have been studied with respect to their anesthetic efficiency and toxicity

A Diethylaminoethyl p-fluorobenzoate
B Dipropylaminoethyl *p*-fluorobenzoate
C Dibutylamino ethyl p-fluorobenzoate
D Diethylamino propyl *p*-fluorobenzoate
E. Dipropylamino propyl *p*-fluorobenzoate
F Dibutylamino propyl *p*-fluorobenzoate

The methods employed to estimate the anesthetic activity of the compounds were limited to three procedures anesthesia of goldfish, anesthesia of the rabbit s cornea, and the dermal wheal, with the intention of extending these if the results seemed to warrant it The goldfish method used was that of Dailey and Benedict (3) This procedure probably does not measure local anesthetic effect exclusively as the systemic effects of the compounds are simultaneously produced by absorption through the gills The rapidity with which the fish became refractory to pinching the tail is in general, however, closely correlated with the anesthetic efficiency of many anesthetics as determined by other methods. Anesthesia of the rabbit s cornea was determined by the method of Schmitz and Loevenhart (4) and the criterion used was the duration of anesthesia since Sinha (5) has pointed out the superior significance of this characteristic. The dermal wheal method used was that of Sollmann (6) and the criterion used was that of duration of anesthesia by a fixed concentration. This is probably less valuable than a determination of the threshold concentration requisite for anesthesia but it is better adapted to provisional experiments The volar surface of the forearm was used and at least four wheals in different subjects were tested for each compound The toxicity of the various compounds was determined

volume and filter To 5 cc of this filtrate add 0 5 cc of 0 1 per cent sodium nitrite and mix. After 3 minutes add 0 5 cc of 0.5 per cent ammonium sulfamate in 1 M sodium dihydrogen phosphate solution and mix After 3 minutes add 0 5 cc of 0 1 per cent N-(1-naphthyl) ethylene diamine dihydrochloride solution and mix After 10 minutes read the solution in the Klett colorimeter using filter #54 This gives the total N^4 hexanoylsulfanilhydroxamide, *while the free or deacylated product is determined by* evaporating 5 cc of the original alcoholic blood filtrate to 1 cc Cool and add 2 cc of *p*-toluenesulfonic acid and make up to 10 cc volume with distilled water Filter and use 5 cc of filtrate for diazotization as described above

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A. Diethylaminoethyl *p*-fluorobenzoate
B. Dipropylaminoethyl *p*-fluorobenzoate
C. Dibutylamino ethyl *p*-fluorobenzoate
D. Diethylamino propyl *p*-fluorobenzoate
E. Dipropylamino propyl *p*-fluorobenzoate
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after subcutaneous injection in mice At least 25 mice were used for each compound and all deaths occurring within 24 hours were included The LD50 was computed by Dragstedt's method (7) The data with respect to anesthetic activity, toxicity and irritation are shown in the table

TABLE 1

Anesthetic efficiency and toxicity of fluorine compounds

	COMPOUNDS							
	Cocaine	Procaine	A	B	C	D	E	F
Onset of anesthesia (minutes) in goldfish*		9 9	9 5	6 3	5 4	8 2	4 6	3 5
Duration (minutes) anesthesia rabbits' cornea†	29		0	3 2	6 0	3 5	7 5	11 0
Degree irritation rabbits' cornea†	0		0	++	++	++	++	++
Duration (minutes) anesthesia human wheal‡		26	21	41 5	32	24 5	53 5	50
Degree irritation—human skin‡		0	0	+++	+++	+	+	+
LD50 mice (mgm per kilogram)		800	2600	2500	7400	2700	1850	2550

* All compounds in 0 5 per cent

† Cocaine HCl in 1 0 per cent, all others 2 per cent

‡ All compounds in 2 0 per cent

DISCUSSION

The activity of the *para*-hydroxybenzoyl alkamine esters prepared by Rohmann and Scheurle (8) encouraged the belief that with the substitution of fluorine for hydroxyl, the alkamine esters of fluorobenzoic acid might retain the anesthetic activity of the *para*-hydroxy esters, but with reduced toxicity The results on six compounds reported here, indicate that this is actually the case All but one of the compounds (A) induced anesthesia in goldfish more rapidly than procaine, elicited a dermal wheal anesthesia of longer duration than procaine, were capable of producing topical anesthesia on the rabbit's cornea, and were less toxic to mice than procaine All of these compounds produced considerable tissue irritation however This was evidenced by marked conjunctival irritation in the rabbit, irritation at the site of the dermal wheals, and irritation to necrosis and sloughing at the site of the subcutaneous injections in the mice Compound A was considerably different from the rest It produced little tissue irritation, and while displaying no substantial capacity to induce topical anesthesia, it appeared to be similar to procaine in both the goldfish and the dermal wheal tests Inasmuch as it appears to be substantially less toxic than procaine it may merit further study

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FURTHER STUDY OF CENTRAL STIMULATION FROM SYMPATHOMIMETIC AMINES

J W SCHULTE, E C REIF, J A BACHER, JR., W S LAWRENCE, AND M. L TAINTER[1]

From the Department of Pharmacology, Stanford University School of Medicine, San Francisco, California

Received for publication October 4, 1940

In addition to effects on the circulation and other systems under autonomic nervous control, certain of the sympathomimetic amines act directly on the central nervous system Although the literature on these compounds is large and rapidly growing, it deals mainly with the autonomic actions, and only limited information is available about the central effects

The stimulus for this study came from certain reactions to these compounds in narcosis produced by various hypnotics (13) It was observed that, although some of these amines had little or no power to hasten awakening from deep narcosis, after the animals were aroused they exhibited excitation resembling intense stimulation of the higher centers of the brain This was characterized by purposeful, coördinated movements resembling those of the excitability of hyperthyroidism and not by convulsive seizures such as occur from stimulation of the medullary centers and spinal cord The activity was of a continuous coördinated type, the animals moving restlessly about the cage, gnawing at the wire, licking their tails, rubbing their noses, and so on The severest form of this activity amounted almost to maniacal frenzy Since this type of stimulation resembled that which may be therapeutically useful in the treatment of narcolepsy and depressed states, it seemed desirable to measure it quantitatively, and to determine, if possible, which compounds possessed an optimal activity Such a study might reveal whether the present clinical popularity of benzedrine (amphetamine) as a central stimulant is justified, and if not, whether a more desirable effect could be obtained with some other amine

A special apparatus for measuring the spontaneous activity of the rats was developed, which would not put them under restraint, or induce activity by itself This has been described elsewhere (12), together with limited observations of certain popular analeptics to serve as controls for the effects of the sympathomimetic amines

[1] Supported, in part, by Grant 380 of the Therapeutic Research Committee of the Council on Pharmacy and Chemistry, American Medical Association, and by the Rockefeller Fluid Research Fund

METHODS

The apparatus used is illustrated in figure 1 It consisted of a small wire cage approximately six inches square holding one rat, suspended from a long flexible spiral spring in such a manner that it moved up and down in response to each movement, or change in balance, of the animal The motion was summated by means of a Harvard work adder connected to the spring, and recorded by having each revolution of the work adder close an electrical circuit through a mercury cup to a signal magnet writing on a long kymograph. Twenty such units were used simultaneously enclosed in a brightly lighted constant temperature chamber kept at between 27 and 29°C These conditions were selected because it is known that white rats have a minimal activity at this temperature, in bright light.

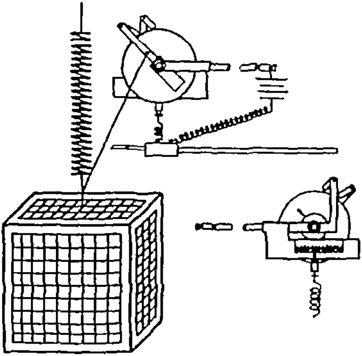

FIG 1 DIAGRAM OF ACTIVITY CAGE AND RECORDING DEVICE

The movements of the cage on the spring are summated by the Harvard work adder This has been modified by the addition of a mercury cup through which the electrical circuit to the signal magnet is closed on each revolution of the adder wheel.

Ten adult rats consisting of 5 males and 5 females, were used as a group each animal being injected subcutaneously with the dose of the drug to be tested in proportion to the body weight,[2] dissolved in not more than 1 0 cc. of fluid. The rats were then quickly placed in the cages and recordings were continued at least four hours, or until the animals had returned to a state of normal activity This usually required between five and seven hours. The results were then read from the kymograph record as revolutions of the work adders in each half hour period. The average activity of the 10 animals for each period was calculated and from these values were subtracted the control values to give the net change in activity

In determining control values, which represented the normal spontaneous activity of unmedicated rats under these conditions we used the method of our first paper (12)

[2] All doses in this paper are expressed in milligrams per kilogram of body weight

One-half of the animals (the controls) were injected subcutaneously with normal saline solution (0 85 per cent NaCl) each day for a considerable period, and the other half with the amine under investigation The following week the injections were repeated with the groups reversed Finally these control observations on 280 individual animals were grouped together and averaged From these values the expected spontaneous activity could be predicted with high probability by the usual statistical methods In this way it was determined that during the first half hour the average control activity of each rat was 2 3 revolutions, during the second half hour 1 0, and during each succeeding half hour 0 8 revolutions Subtracting these "blank" values from those of the corresponding experimental periods, we obtained the net change of activity ascribable to the drug injected The summated activity over the first 4 hours in the controls averaged 8 1 revolutions for each rat, with a standard error of the mean of 2 57 revolutions for groups of 10 rats Definite stimulation was not assumed to be produced unless the medicated rats exceeded this basic control activity by at least 3 standard errors or 7 7 revolutions total in the first 4 hours

The doses used began with amounts of the amines which were completely ineffective and were increased by increments of 100 per cent up to 320 mgm. per kilo, unless either convulsions or death ensued at a lower dosage level With a few of the amines, the complete range of dosage could not be tested because of limited solubility or insufficient available amounts of the compounds No group of rats was used more frequently than once a week, and usually, at least 10 days intervened between successive tests

The amines investigated included all the available members of the sympathomimetic group, as the racemic hydrochlorides, unless otherwise indicated The compounds consisted mainly of variants on the phenyl-ethyl and phenyl-propyl amine structures, with a few aliphatic amines and other derivatives Among these were ephedrine, benzedrine, and epinephrine, and their optical isomers Some less closely related com pounds, such as *naphtholethylamine, were also included*[1]

RESULTS

The names of the compounds and the results obtained are summarized in table 1, according to chemical group, range of dosage tested, threshold stimulant dose, maximum peak and total effect, therapeutic margin and fatal dose Because of the large number of compounds studied it is not feasible to discuss the results in any except general terms However, sufficient detail is given in table 1 to permit specific conclusions regarding each compound The average responses to *d*-benzedrine, a potent compound, are summarized in figure 2, which indicates the speed of onset, intensity, and duration of action at various dosage levels

[1] The compounds listed below were supplied gratuitously by the manufacturers indicated, the remainder being obtained through the usual commercial sources (The numbers correspond with the listings in Table 1) Abbott Laboratories, *20, 21,* Dr G A Alles, *2, 3, 4, 5,* Professor George Barger, *23, 27, 29, 30, 37, 38, 65, 66,* Bilhuber Knoll Corp , *74,* Burroughs Wellcome & Co , *62,* Professor A C Crawford, *26,* Hoffman La Roche, Inc , *34,* I G Farbenindustrie Ges , *42, 50, 51, 54,* Eli Lilly & Co , *22,* Merck & Co , *31,* H A Metz & Co , *57,* Dr Armando Novelli, *24,* Sharp & Dohme, Inc , *6, 7, 8, 25, 28, 32, 33, 35, 36, 41, 52, 53, 55, 59, 61,* Smith, Kline & French, Inc , *9, 10, 11, 12, 13, 14, 15, 16, 40, 43,* Frederick Stearns & Co , *44, 45, 47, 48, 49,* Professor Trefouel, *19,* Winthrop Chemical Co , *58, 60, 63, 64, 67, 68, 69*

TABLE 1

The central stimulant activity in unnarcotised white rats of sympathomimetic amines injected subcutaneously

All doses are expressed in milligrams per kilogram of body weight. In the fatal dose column are shown the lowest lethal dose and proportion of animals killed. Therapeutic margin is the fatal dose divided by the threshold dose

Number	Formula	Name	Range of doses	Threshold stimulant dose	Maximum peak effect: Dose	Maximum peak effect: Revolutions per hour	Maximum total effect: Dose	Maximum total effect: Total Revolutions	Maximum total effect: Duration	Fatal dose and proportion of animals killed	Therapeutic margin
	Phenyl-ethyl derivatives										
			mgm.	mgm.	mgm.		mgm.		hours		
1	Phenyl-*1*-amino-*2*-ethane	Phenylethylamine	10-160	80	80	22.2	160	19 1	4	160, 1/10	2.0
2	Phenyl-*1*-methylamino-*2*-ethane	Phenylethylmethylamine	40-320	160	160	14 4	160	9 1	4		
3	Phenylethylguanidine		40-80	80	80	27.2	80	24 1	4		
4	Phenyl-*1*-amino-*2*-ethanol-*1*	Phenylethanolamine	40-320	320	320	26.8	320	42 9	5	320, 3/10	1.0
5	Phenyl-*1*-acetylamino-*2*-ethanol-*1*		40-640	640	640	5 6	640	18 0	6		
	Phenyl-propyl derivatives										
6	Phenyl-*1*-amino-*1* propane		5-160	0						160, 3/7	
7	Phenyl-*2*-amino-*1*-propane		0.2-80	0							
8	Phenyl-*3*-amino-*1*-propane		5-10	0							
9	*dl*-Phenyl-*1*-amino-*2*-propane SO_4	*dl*-Benzedrine	0 04-20	0.3	2 5	81.2	20	310 4	6	10, 1/10 20 5/10	33.3
10	*d*-Phenyl-*1*-amino-*2*-propane SO_4	*d*-Benzedrine	0.06-10	0.125	2.5	90.2	5	234.3	7	10, 1/10	80 0
11	*l*-Phenyl-*1*-amino-*2*-propane SO_4	*l*-Benzedrine	0 5-160	2.5	10	47.2	20	134.3	7	160, 3/10	64.0
	Phenyl-propanol derivatives										
12	Phenyl-*1*-amino-*2*-propanol-*1*	Propadrine	5-80	40	80	30 4	80	99 7	4	80, 1/10	2 0
13	Phenyl-*1*-amino-*2*-butanol-*1*	Butadrine	20-320	0							
14	Phenyl-*1*-amino-*2*-pentanol-*1*	Pentadrine	20-320	320	320	18.0	320	27 7	4		
15	Phenyl-*1*-amino-*2*-hexanol-*1*	Hexadrine	20-80	0							
16	Phenyl-*1*-amino-*2*-octanol-*1*	Octadrine	20-80	0							

TABLE 1—*Continued*

NUMBER	FORMULA	NAME	RANGE OF DOSES	THRESHOLD STIMULANT DOSE	MAXIMUM PEAK EFFECT		MAXIMUM TOTAL EFFECT			FATAL DOSE AND PROPORTION OF ANIMALS KILLED	THERAPEUTIC MARGIN
					Dose	Revolutions per hour	Dose	Total Revolutions	Duration		
	Ephedrine isomers (phenyl 1 methylamino-2 propanol 1)										
			mgm	*mgm*	*mgm*		*mgm.*		*hours*		
17		*dl* Ephedrine	2 128	16	128	33.0	128	171.3	8	128 2/10	8
18		*l* Ephedrine	2 5–160	5.0	20	85.6	20	186.8	6	160 5/10	32.0
19		*d* Ephedrine	5–160	40	160	33.0	160	113.5	7	160 1/10	4.0
20		*l* Pseudo-ephedrine	10 160	20	160	53.0	160	313.7	11	160 2/10	8.0
21		*d* Pseudo-ephedrine	10–80	20	80	65.4	80	181.3	14	80 1/10	4.0
22	*l* Phenyl 1-dimethyl amino-2 propanol 1	*l* Methyl ephedrine	40–640	80	640	14.4	640	11.8	7		
	Diphenyl derivatives										
23	Diphenylethyl-amine		10–40	20	20	7.2	20	9.2	4		
24	*1-3* Diphenyl-amino-2 propane		40 160	0							
25	Diphenylethanol amine		40–80	0							
	Substituted phenyl ring derivatives										
26	*p*-Methoxy phenylmethyl amine		5–320	0							
27	*p*-Chlorphenyl 1 amino-2-ethane		5–40	0							
28	*p*-Chlorphenyl 1-amino-2 propanol 1		10–160	160	160	9.9	160	37.9	6	160 5/10	1.0
29	*p*-Nitrophenyl 1-amino-2 ethane		5 20	0							
30	Ethyl ester of *p*-carboxy phenyl-ethylamine		5–40	0							
31	*p*-Aminophenyl 1 methyl-amino-2 propanol 1	Ephetonal	5–160	160	160	7.2	160	9.4	4	160 1/10	1.0
32	*p*-Methoxyphenyl-1 amino-2 propanol 1		5 160	0						160 5/10	
33	*2 4* Dimethoxy phenyl 1 amino-2 propanol 1		5–320	5	5	3.8	5	8.8	4	320 3/10	64.0
34	*3-4-5*-Trimethoxyphenyl 1-amino-2-ethane	Mescaline	5–320	10	320	24.2	320	75.3	6	320 5/10	32.0
35	*p*-Tolyl 1 amino-2 propanol 1		10–80	80	80	16.8	80	14.6	4	80 1/10	1.0
36	*m*-Tolyl 1 amino-2 propanol 1		5–40	40	40	10.4	40	20.0	4	40 6/10	1.0
37	Thiophenethylamine		5–320	10	160	15.4	40	26.0	6	320 7/8	3[illegible].0
38	Naphtholethylamine		5 20	0							
	p-Hydroxyphenyl ring derivatives										
39	*p*-Hydroxyphenyl 1 amino-2 propane	Tyramine	5–320	0							
40	*p*-Hydroxyphenyl 1 amino-2 propane HBr	Paredrine	5–320	80	320	60.6	320	98.5	6	320 1/10	4

TABLE 1—*Continued*

NUMBER	FORMULA	NAME	RANGE OF DOSES	THRESHOLD STIMULANT DOSE	MAXIMUM PEAK EFFECT		MAXIMUM TOTAL EFFECT			LETHAL DOSE AND PROPORTION OF ANIMALS KILLED	THERAPEUTIC INDEX
					Dose	Revolutions per hour	Dose	Total Revolutions	Duration		
	p-Hydroxyphenyl ring derivatives—Continued										
			mgm.	mgm.	mgm.		mgm.		hours		
41	p-Hydroxyphenyl-1 amino-2-propanol-1		5-160	0							
42	p-Hydroxyphenyl-1 methylamino-2-propanol-1	p-Oxyephedrine	5-32	0							
43	p-Hydroxyphenyl-1 methylamino-2-propane SO_4	Paredrinol	5-320	160	160	15.5	160	48.5	4	320, 9/10	3
44	l-p-Hydroxyphenyl-1 methylamino-2-ethanol-1	Synephrine	5-320	0						320, 2/10	
45	p-Hydroxymethylamino-acetophenone	Synephrine Ketone	10-320	0							
46	p-Hydroxyphenyl-1-dimethylamino-2-ethane	Hordenine	10-160	0							
	o- and m-Hydroxyphenyl ring derivatives										
47	l-m-Hydroxyphenyl-1 methylamino-2-ethanol-1	Neosynephrine	2.5-20	0						10 4/10 20 2/10	
48	d-m-Hydroxyphenyl-1 methylamino-2-ethanol-1	d-Neosynephrine	10-160	0							
49	m-Hydroxymethylamino-acetophenone	Neosynephrine Ketone	10-320	160	160	33.2	320	38.7	3	320, 2/10	2
50	l-m-Hydroxyphenyl-1 methylamino-2-propanol-1	l-m-Oxy ephedrine	5-10	0						10, 2/10	
51	m-Hydroxyphenyl-1-amino-2-propanol-1	m-Oxynor ephedrine	5-80	0						40, 1/10 80, 4/20	
52	m-Hydroxyphenyl-α-aminoethylketone		10	0							
53	(3-Hydroxy-4-methylphenyl)-1-amino-2-propanol-1		2.5-5	5	5	14	5	13.6	3	5, 1/10	1
54	o-Hydroxyphenyl-1-methylamino-2-propanol-1	o-Oxyephedrine	10-160	0							
55	o-Hydroxyphenyl-1 amino-2-propanol-1		10-80	0							
	Dihydroxyphenyl ring derivatives										
56	l-(3-4-Dihydroxyphenyl)-1-methylamino-2-ethanol-1	Epinephrine	0.13-4	0.25	2.0	13.6	2.0	19.2	2	4, 5/10	16
57		d-Epinephrine	5-40	0						40, 5/10	

TABLE 1—*Concluded*

NUMBER	FORMULA	NAME	RANGE OF DOSES	THRESHOLD STIMULANT DOSE	MAXIMUM PEAK EFFECT		MAXIMUM TOTAL EFFECT			FATAL DOSE AND PROPORTION OF ANIMALS KILLED	THERAPEUTIC MARGIN
					Dose	Revolutions per hour	Dose	Total Revolutions	Duration		
			Dihydroxyphenyl ring derivatives—*Continued*								
			mgm	*mgm*	*mgm*		*mgm*		*hours*		
58	*3-4* Dihydroxymethyl-aminoacetophenone	Epinephrine Ketone	0 5-80	0						80 1/10	
59	(*2-4* Dihydroxyphenyl) *1*-amino-*2* propanol *1*		0 5-5	0							
60	(*3-4* Dihydroxyphenyl) *1*-amino-*2*-ethanol-*1*	Arterenol	0 25-2	0						2 1/10	
61	*3 4*-Dihydroxyphenyl-α-aminoethyl ketone		2 5-40	40	40	8 0	40	9 9	2		
62	(*3-4* Dihydroxyphenyl) *1* methylamino-*2* ethane	Epinine	0 25-160	80	80	16	80	13 0	2	160 2/10	2
63	(*3-4*-Dihydroxyphenyl) *1*-amino-*2* propanol *1*	Cobefrine	0 25-5	0						2 5 2/20 5 0 1/10	
64	(*3-4* Dihydroxyphenyl) *1 methylamino-2 pro*-panol-*1*	*3-4* Dioxy *ephedrine*	1-40	0						40 1/10	
65	(*3-4* Dihydroxyphenyl) *1*-amino-*3*-propane		2 5-40	0							
66	(*3-4*-Dihydroxyphenyl) *1*-amino-*2*-ethane		2 5-40	0							
67	(*3-4*-Dihydroxyphenyl) *1* amino-*2*-butanol *1*	Ethylnor suprarenin	1-160	0						160 6/10	
68	(*3-4* Dihydroxyphenyl) *1*-ethylamino-*2*-ethanol *1*		0 5-40	0						40 4/10	
69	(*3-4*-Dihydroxyphenyl)-*1* methylamino-*2*-butanol *1*		5-160	0						*160 1/10*	
			Aliphatic and other derivatives								
70	*n* Amylamine		5-320	10	320	9 6	320	8 2	2	320 1/10	32
71	*Isopropylamine*		*5-320*	*0*							
72	Secondary butylamine		20-320	0							
73	Isobutylamine		10-320	0							
74	Methylamino-methyl heptene	Octin	10-160	0						160 10/10	
75	Benzylmethylamine		10 320	80	320	8 6	80	7 0	3		

It is seen that, according to minimum effective or threshold doses, the most active compounds, in descending order of activity, were as follows *d*-benzedrine, *l*-epinephrine, *dl*-benzedrine, *l*-benzedrine, *l*-ephedrine, *2-4*-dimethoxyphenyl - *1* - amino - *2* - propanol - *1*, (*3* - hydroxy - *4* - methylphenyl) - *1* - amino-*2*-propanol-*1*, mescaline, thiophenethylamine, and n-amylamine All the other compounds required over 10 mgm per kilo to produce any excitation The *d*- and *dl*-benzedrine and *l*-epinephrine were in a unique position,

since the threshold doses of less than 0 5 mgm indicated an order of cerebral activity much greater than that of all the other compounds. Also worthy of special comment, because of their wide clinical usage, were synephrine (sympatol), neosynephrine, and cobefrine, which did not cause central stimulation in any tolerated dose

Another useful measure of excitation is the maximum degree of stimulation that a compound can cause at optimum dosage Under the conditions used by us the maximum stimulation compatible with life was about 85 revolutions per hour at which level the animals exhibited almost maniacal frenzy Such intensity of stimulation was produced by *d*- and *dl*-benzedrine

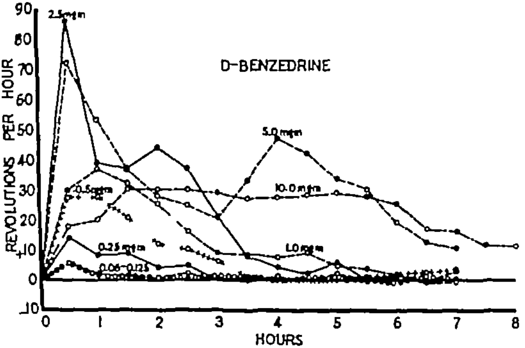

FIG 2 CHANGES IN SPONTANEOUS ACTIVITY PRODUCED BY VARIOUS DOSES OF *d* BENZEDRINE WHEN INJECTED SUBCUTANEOUSLY IN WHITE RATS

Each curve shows the average net increase in activity of 10 rats on a given dose above that of the control animals.

and *l*-ephedrine Almost as great an intensity was shown by *d*-pseudoephedrine and paredrine while *l* pseudoephedrine, propadrine and *l*-benzedrine were slightly less active causing a maximum of about 50 revolutions per hour The phenylethyl-derivatives and the remaining ephedrines stimulated still less intensely i.e., about 15 to 30 revolutions per hour A considerable number of the remaining amines showed some though unimportant, activity Despite its low threshold dose *l*-epinephrine caused only 13.8 revolutions per hour as a maximum peak effect.

Differences in the duration of action of the various compounds were noted for some produced sharp increases in activity of short duration, while others

exhibited more gradual effects which were more persistent The increase in the average total number of revolutions over the entire period of an amine's action summated the total amount of stimulation produced *dl*-Benzedrine and *l*-pseudoephedrine were practically identical in this respect, both causing an average total increase of about 310 revolutions in 11 and 8 hours, respectively *d*-Benzedrine fell to a definitely lower level of 234 revolutions in 7 hours, and still lower were other ephedrines, ranging between 113 and 186 revolutions total increase Paredrine and propadrine, with about 100 revolutions total, were the least effective of this more potent group Because of a lesser intensity of the peak effect, and a short duration of three hours, *l*-epinephrine caused a total average increase of only 19 2 revolutions after the most effective dosage, which was 2 mgm

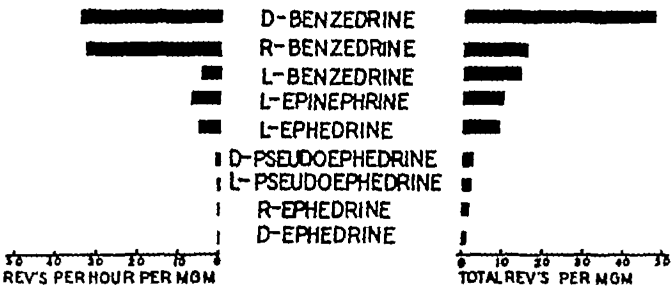

FIG 3 RELATIVE CENTRAL STIMULANT ACTIVITY OF THE MORE EFFECTIVE COMPOUNDS
The blocks on the left side indicate the maximum degree of stimulation produced by the most effective dose per milligram per kilogram body weight of drug administered and the blocks on the right side, the greatest total stimulation per milligram per kilogram administered The calculations were made from the averages of groups of 10 rats on each dose

Probably a clearer impression of the relative potency of these compounds can be obtained from the amounts of stimulation produced per milligram of drug administered On this basis, the outstanding potency of benzedrine is even more apparent *d*-Benzedrine produced an average peak response of 34 6 revolutions per milligram and an average total increase of 47 revolutions per milligram, for the most effective dosage *l*-Benzedrine caused only 4.8 peak revolutions and 13 4 total revolutions increase per milligram, while *dl*-benzedrine was intermediate between the two, as would be expected *l*-Ephedrine and *l*-epinephrine were of the same order of activity as *l*-benzedrine The relative potency of these compounds is indicated in figure 3 The blocks show strikingly the sharp decrease in activity which occurs with deviations from the benzedrine structure

No attempt was made to determine accurately the fatal doses, but when deaths occurred they were recorded for the information they gave on the

probable order of magnitude of these values. *d* Benzedrine *killed one rat* out of 10 on the 10 mgm. dosage, while the racemic compound did the same in addition to 5 out of 10 rats on the 20 mgm dosage *l* Benzedrine was much less toxic since 100 mgm was required to kill any of the rats Scattered deaths occurred with propadrine at the 80 mgm. level, and with the ephedrines *between 80* and *100* mgm. *Among the other important* compounds, neosynephrine killed at a 10 mgm. dose, epinephrine at 4 mgm. and cobefrine at 2.5 mgm

The significance of these fatal doses is mainly in comparison with the dose required to produce stimulation Dividing the fatal dose by the threshold central stimulant dose gives a value which may be used to express the margin of safety of the compound as a central stimulant. These values are presented for the active compounds, in the last column of table 1 *d* Benzedrine, with a value of 80 clearly had the greatest margin of safety, while the *levo* form had a value of 64 and the racemic 33 *l*-Ephedrine had practically the same value as *dl*-benzedrine i.e 32 The remaining efficient compounds showed much narrower margins, i e, *l*-epinephrine 16 *l*-pseudoephedrine and *dl* ephedrine 8 *d* pseudoephedrine 4, and propadrine only 2

When the relation of chemical structure to stimulant potency is considered, it is obvious that the most effective arrangement of the molecule was phenyl-*1*-amino-*2* propane. When the relative positions of the amino and phenyl groups on the propyl side chain were changed, the activity was completely lost. Shorter side chains, as in the ethyl derivatives and longer ones as in the butyl compounds were also generally less effective. Addition of an alcoholic radicle on the first carbon of the side chain decreased the activity of benzedrine but did not abolish it. Substitution of a methyl group on the *amino radicle variably effected the stimulant efficiency of the compound.* When methoxy, chlor, amino, or methyl groups were introduced into the phenyl ring the activity was greatly weakened or abolished Thiophen substituted for the phenyl ring, as in thiophenethylamine, was about as active as the corresponding phenylethylamine, but had a considerably lower threshold dose. Presence of a naphthol ring completely abolished the activity Addition of a second methyl group to the amino group decreased the potency of the amines Substitution of one hydroxy group in the phenyl ring decreased or abolished the potency, but two hydroxy groups as in epinephrine increased it markedly Whether this was generally true could not be determined, since the marked circulatory effects of these catechol derivatives restricted the range of dosage which could be tolerated. Of 5 straight chain compounds investigated, only N-amylamine was found to possess even slight activity

Generalizations can not be made as to the effect of optical rotation on analeptic potency *d* Benzedrine was the most active compound studied, surpassing considerably its *levo* isomer but in other groups, *l*-epinephrine and

l-ephedrine were more active than their corresponding *dextro* isomers, while among the pseudoephedrines optical isomerism made little difference

DISCUSSION

The true site or mechanism of the central actions of the sympathomimetic amines studied has not yet been determined Morita (10), in 1915, found that ephedrine, among other compounds, aroused decorticate dogs from light chloral narcosis Accordingly, he placed the site of action as being subcortical, and somewhere near the hypothalamus Others, including ourselves, have investigated the effects of certain of these compounds on the action of various hypnotics, particularly the barbituric acid derivatives, chloral hydrate, paraldehyde and avertin (13, 11) Jacobsen (1, 8) studied several derivatives of phenylisopropylamine, ephedrine and β-phenylethylamine, and found that all save the latter had antagonistic effects against hypnotics when the narcosis was light Alles (6) reported similar results for benzedrine, when given to chloralized rabbits Hjort (7) *et al* found picrotoxin and metrazol better than benzedrine in combating barbital narcosis, and somewhat safer to use

In this laboratory (13), it was found that the compounds most effective in restoring reflex responses under deep narcosis of avertin, chloral, or pentobarbital, were those belonging to the group of phenylisopropylamines Practically essential were an amino group in the *2* position and the phenyl ring on the first carbon atom This agreed with the results of Jacobsen and his coworkers, and with the results of this study of unnarcotized animals Jacobsen also found that introduction of an hydroxy group in the side chain attenuated the action, which also was true for the undepressed rats used by us, except that the ephedrine compounds, with their hydroxy group in the side chain, still had considerable potency

Other investigators (5, 9) have observed that there is no correlation between the sympathomimetic effects and analeptic potency of these amines, which is also amply clear from the results in this report Gunn and his coworkers (3) reported that with the same phenyl nucleus the iospropylamine side chain made the compound more toxic, though more stimulating to the central nervous system, and less sympathomimetic in action than the corresponding ethylene side chain Alles (4, 6) found the optical isomers of phenylisopropylamine to be effective as analeptics He decided, from the subjective impressions of three human subjects, that *d*-benzedrine was four times as active as the *l*-isomer, and twice as active as the racemized compound In this study, conducted on animals by objective methods, it was found that *d*-benzedrine was about three times as effective as the *l*-isomer in terms of milligram activity, and nearly one and one-half times as active as the racemized compound

Jacobsen (2) observed that benzedrine increased mental activity in man but tended to be less effective in individuals reluctant to take part in the test, thus indicating a factor of suggestion. Many such observations have been made on the psychological effects of benzedrine in both animals and man. There is general agreement that certain varieties of central stimulation can be produced, but this is not uniform for all manifestations of cerebral activity There is, in addition, adequate clinical evidence that both ephedrine and epinephrine also have some such effects. These actions were apparent in this study and have now been given a quantitative evaluation as to the relative intensity and relationship to tolerated doses

SUMMARY AND CONCLUSIONS

1. A method is described for the quantitative determination of the spontaneous activity of unnarcotized unrestrained rats, which has been applied to a study of the central stimulant actions of a group of 75 sympathomimetic amines injected subcutaneously The doses used ranged from the completely ineffective to the convulsant or fatal.

2 The phenylisopropylamines were considerably more effective than the other amines as regards minimum effective dosage degree of stimulation, and intensity of effect per milligram of amine The *dextro* isomer of phenyl isopropylamine (*d*-benzedrine) was the most active form of this molecule

3 The various optical isomers of ephedrine had some central stimulant power, but only of a magnitude approaching that of the weaker benzedrine isomer the *levo* derivative. *l*-Ephedrine was the most potent of this group while the pseudoephedrines were less active.

4. *l* Epinephrine caused definite stimulation in low doses comparable to those of *d* benzedrine. However the intensity and duration of response were much less than with the phenylisopropylamines in those doses which could be tolerated by the circulatory system.

5 No central stimulation was produced by such clinically effective compounds as tyramine synephrine (sympatol) neosynephrine (adrianol) adrenal one (kephrine), cobefrine (corbasil) and octin in maximum non fatal doses.

6. Changing the length of the side-chain, rearrangement of the relative positions of the groups in phenylisopropylamine, or substitution of hydroxy chlor amino methoxy or methyl groups in the phenyl ring did not increase the central stimulant efficiency of the compounds.

7 The group of drugs studied was extensive enough to demonstrate that the optimum central stimulation resided in the *d*-phenylisopropylamine structure, and that all deviations from this configuration were accompanied by sharp reductions in potency Therefore there would appear to be little probability that a more effective compound could be developed within this group of amines than the compounds already available

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PHARMACOLOGICAL STUDIES IN EXPERIMENTAL ALCOHOLISM

I THE EFFECT OF SYMPATHOMIMETIC SUBSTANCES ON THE BLOOD-ALCOHOL LEVEL IN MAN[1]

MAX RINKEL AND ABRAHAM MYERSON

From the Division of Psychiatric Research, Boston State Hospital, Boston, Mass

Received for publication June 13, 1940

Many researches have concerned themselves with attempts to modify the alcohol concentration of the blood by pharmacological methods With the exception of the work of Fleming and Reynolds (1), however, no attempt has been made to alter the curve of the blood-alcohol concentration by sympathomimetic drugs. These investigators used adrenalin which they found to produce no significant effect on the alcohol concentration in the blood when the alcohol was injected intravenously The chemical observations which led Reifenstein and Davidoff (2) to conclude that amphetamine (benzedrine) sulfate has a favorable effect on acute alcoholic intoxication, and the work of Bloomberg (3), which strongly indicates that this drug favorably influences the treatment of chronic alcoholism, suggested to us that amphetamine sulfate might act by diminishing the amount of alcohol in the circulation as well as by its generally recognized effect in stimulating the central nervous system and the experiments to be reported below were intended to elucidate this point

The following drugs were studied Amphetamine sulfate B-para hydroxyphenylisopropylamine (paredrine) adrenalin chloride,[2] and atropine sulfate (Sharp and Dohme) The results of these studies are here reported.

MATERIALS AND METHODS

Fifteen patients were selected for this study With the exception of one patient, who was a chronic alcoholic, but who has not used alcohol for the many years that he has been an inmate of this hospital the patients were schizophrenics in the various stages of that disease and were well known to us for several years in respect to their

[1] Aided by grants from the Commonwealth of Massachusetts, the Rockefeller Foundation and the Works Progress Administration Projects No 18088 and 21176

[2] The following preparations were used Amphetamine sulfate (Smith Kline & French) tablets of 10 mgm and ampoules of 20 mgm. dissolved in 1 cc. sterile physiologic saline solution

Paredrine (Smith Kline & French) ampoules of 20 mgm. (1 cc) in sterile water
Adrenalin chloride (Parke Davis & Co) ampoules of 1 cc (1 1000)

physical condition and reaction to autonomic drugs After a 12 to 16 hour fasting period the patients came to the laboratory in the morning without breakfast With the exception of one patient who had received 0 66 grams of ethyl alcohol per kilogram body weight, the dosage was 0 5 grams of ethyl alcohol per kilo Alcohol was administered orally, intravenously or duodenally For the oral and duodenal administration the required amount of alcohol was made up to a volume of 100 cc with water In the case of the former, the alcohol was given in an ice-cooled solution. For the latter the following technique was used Under continuous fluoroscopic control a duodenal tube was introduced into the duodenum, alcohol was then injected through this tube, precaution being taken to prevent the tube from slipping back into the stomach, a mishap that frequently follows the use of the duodenal tube For intravenous injection a standard solution of 20 per cent alcohol (by volume) in physiological saline was made up under the customary sterile conditions and the alcohol was slowly injected by gravity over a period of from three to eight minutes

Before an experiment was performed 3 to 5 control tests were made on the same subject Blood samples from the basilic vein were taken before and at 30, 60, 90 and 120 minutes after the administration of alcohol, in some instances samples were also taken simultaneously from the brachial artery In a number of cases blood samples were also withdrawn at 5, 10, 20, 30, 40, 60, 90 and 120 minute intervals The alcohol curves thus obtained at different times on the same subject were essentially the same in height and slope The experiment itself, which took place three to five days after the control test on the same subject, was identical with the control in all respects except for the addition of the administration of the drug whose influence upon the blood-alcohol concentration was to be determined

In each individual the systolic and diastolic blood pressure readings and pulse rate were recorded so as to give an index of the effectiveness of the drug and the sensitivity of the subject to it All the patients responded to the administration of amphetamine sulfate, paredrine, and the intravenous injection of adrenalin by a marked rise of systolic blood pressure In one instance the patient was over-sensitive to 20 mgm of paredrine as manifested by his marked complaints of headache and persistent vomiting. With the exception of this one case, no untoward effects following the administration of the drugs were observed In every instance the blood samples were immediately examined

The method used to determine the alcohol content of the blood was a combination of the methods reported by Fleming and Stotz (4) and by Gibson and Blotner (5) It is based on the principle of the reduction of potassium dichromate in dilute sulfuric acid and consists of three main parts *1* a preparation of the blood sample by the Folin-Wu method which causes precipitation of both serum protein and hemoglobin, *2* distillation of the filtrate into a cylinder containing dichromate-sulfuric acid solution, and *3* determination of the alcohol content by direct measurement of the diminution of the color of potassium dichromate resulting from its reduction by alcohol. The determination was made with an Evelyn photoelectric colorimeter Special care was taken in cleaning and drying the glassware, the technique of Schwarz was used (11), with this exception, that we employed the usual dichromate cleaning solution instead of Schwarz's fuming nitric acid. All connections were made of ground glass and no rubber connections were used Each condenser was constructed in such a way that its lower end was so long that it could be immersed into the dichromate solution of the receiver

RESULTS

1 *Effect of amphetamine sulfate on the concentration of alcohol in blood*

Amphetamine (benzedrine) sulfate was used in 17 experiments in amounts ranging from 30 to 40 mgm intravenously and from 20 to 40 mgm orally

This drug was effective in all experiments, the result being a marked depression of the alcohol concentration in the blood. This is well demonstrated by figure 1, which shows the blood-alcohol curve obtained with the intravenous injection of 40 mgm. of amphetamine sulfate following within one minute the oral administration of 0.66 grams of alcohol per kilo, with the normal blood-alcohol curve of this subject as a control. It will be seen that the inhibition of the alcohol concentration is greatest in the first 30 minute interval, then the inhibition gradually diminishes, but is still present and of considerable height at the end of the 120 minutes of the experiment.

In 9 subjects who received 0.5 grams of alcohol per kilo and 40 mgm. of amphetamine sulfate, the depression of the alcohol concentration of the blood

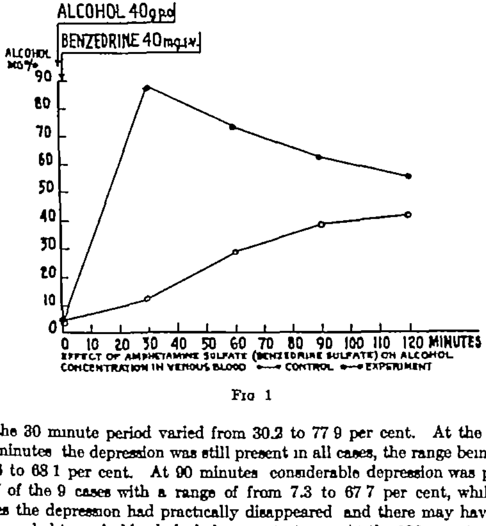


FIG. 1

at the 30 minute period varied from 30.2 to 77.9 per cent. At the end of 60 minutes the depression was still present in all cases, the range being from 24.6 to 68.1 per cent. At 90 minutes considerable depression was present in 7 of the 9 cases with a range of from 7.3 to 67.7 per cent, while in 2 cases the depression had practically disappeared and there may have been been a slight rise in blood-alcohol concentration. At the 120 minute period only 4 of the 9 cases showed depression of the blood-alcohol level the range being from 37 to 55.7 per cent, while in 5 cases there was no depression but an excess of alcohol concentration ranging from 3.1 to 36.2 per cent over the concentration found at this time in the control studies.

When 30 mgm. of amphetamine sulfate was used, the results paralleled

the above figures, although the depression of blood-alcohol level was somewhat less In 2 experiments, amphetamine sulfate was given orally Here also there was an inhibition of blood-alcohol concentration In the experiment in which 20 mgm was given by mouth, there was some inhibition of the alcohol concentration at the thirty minute period, but this was relatively brief as compared to that of the 40 mgm. oral dose, which is almost equal in its effect to that of the same dose given intravenously

The amphetamine sulfate effect in respect to the level of the blood-alcohol seemed most marked in subjects sensitive to the former drug, and this effect

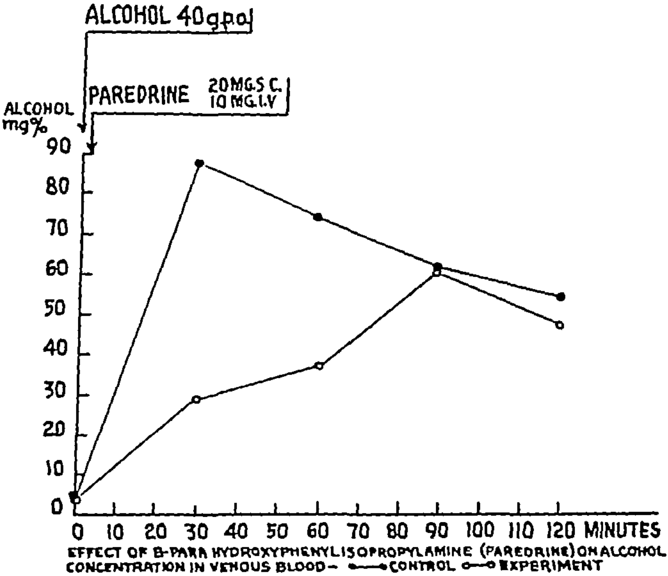


FIG 2

is most prolonged and greatest in those individuals who normally show a lesser and slower uptake of the alcohol into the circulating blood

2 Effect of B-parahydroxyphenylisopropylamine (paredrine) (figure 2)

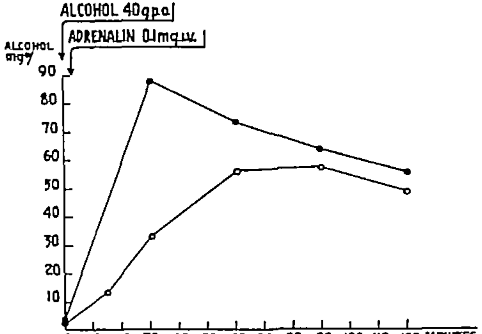
Paredrine was used in 9 experiments, the amount administered varying from 10 to 40 mgm intravenously, with the exception of one patient in whom 20 mgm subcutaneously and an additional 10 mgm intravenously were given The alcohol was administered in exactly the same way as in the amphetamine experiments Without going into detail, it may be stated that the effect of paredrine on the alcohol concentration of the blood was very similar to that

of amphetamine sulfate. However, the duration of the paredrine effect was shorter and the decline of the alcohol inhibition in the blood more rapid.

3 Effect of adrenalin chloride (figure 3)

The results obtained with amphetamine sulfate and paredrine made it likely that the underlying factor was adrenergic in nature Therefore adrenalin, as the classical representative of this group of drugs, was applied in another series of experiments

Five experiments were carried out in which the drug was administered as follows In one instance, 0 1 mgm was given intravenously in another 0.2



EFFECT OF ADRENALIN CHLORIDE ON ALCOHOL CONCENTRATION IN VENOUS BLOOD ○——○ CONTROL ●——● EXPERIMENT

FIG 3

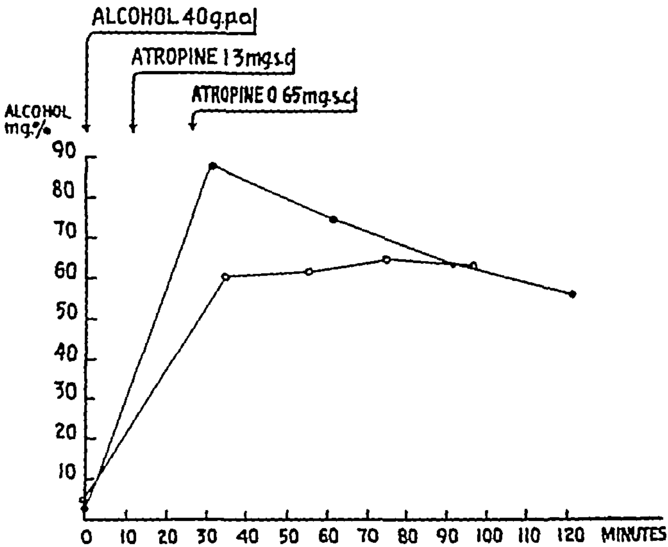
mgm. were dissolved in 10 cc. of water and slowly injected intramuscularly over a period of ten minutes In three instances 2 mgm of adrenalin dissolved in 1 cc of peanut oil were injected intramuscularly This latter combination was used because it effects a slow absorption of adrenalin and thus avoids a sudden rise in blood pressure and the usual uncomfortable side-reactions

Figure 3 shows the effect of the intravenous injection of 0 1 mgm. of adrenalin While the diminution of alcohol concentration in the blood at the thirty minute interval was still considerable at the sixty minute interval this diminution was only moderate, and at the ninety minute interval the blood concentration level almost equaled that of the control curve This

qualitative effect of adrenalin was present in all our experiments regardless of the mode of administration, quantitatively, however, the intravenous injection was the most effective one

4 Effect of atropine sulfate (figure 4)

In 3 cases, 2 6 mgm of atropine were administered subcutaneously, and in one case 1 3 mgm of atropine were given subcutaneously together with 20 mgm of amphetamine sulfate intravenously The increased pulse rate was used as an indication of the effectiveness of the drug, repeated doses of 0 65



EFFECT OF ATROPINE SULPHATE ON ALCOHOL CONCENTRATION IN VENOUS BLOOD •—• CONTROL o—o EXPERIMENT

FIG. 4

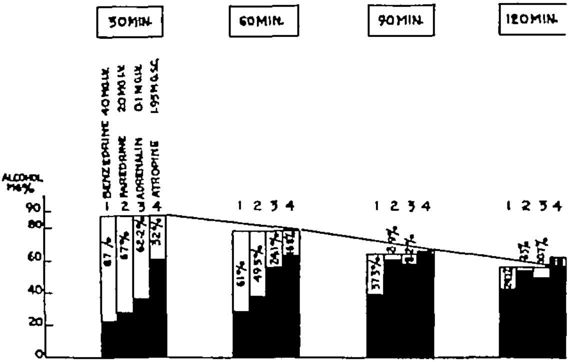
mgm being given until the pulse rate was raised to that point at which an added dose of atropine sulfate did not increase it further

As figure 4 shows, atropine sulfate also depresses the blood-alcohol concentration The percentage of inhibition at the 30 minute interval ranged from 31 9 to 80 5 per cent At the 60 minute interval the range was from 16 8 to 59 5 per cent From the 90 minute period on inhibition began to disappear Although the data indicate a great individual variation, this variation, as in the case of amphetamine and paredrine, seemed to be dependent on the sensitivity of the individual to the drug as measured by the increase of the pulse rate

5 Comparative effect of amphetamine sulfate paredrine, adrenalin and atropine sulfate (figure 5)

The standard of effectiveness of these drugs (with the exception of atropine sulfate) was the rise of systolic blood pressure, and in order to compare their effect on the blood-alcohol level we used doses sufficient to produce about an equal rise in systolic blood pressure. The effect on the blood-alcohol level, however, varied considerably

COMPARATIVE EFFECT OF CLINICALLY EQUIVALENT AMOUNTS OF ADRENERGIC SUBSTANCES ON ALCOHOL CONCENTRATION IN VENOUS BLOOD



RESULTS OBTAINED IN SAME PATIENT WHO ORALLY RECEIVED 0.66 GM. OF ALCOHOL PER KGM. BODY WEIGHT EACH RECTANGLE INDICATES CONTROL ALCOHOL LEVEL – THE BLACK SPACES INDICATE ALCOHOL CONCENTRATION FOLLOWING DRUG ADMINISTRATION. THE WHITE SPACES INDICATE PERCENTAGE OF INHIBITION.

FIG 5

As will be seen in figure 5 amphetamine is the most effective drug in depressing the blood-alcohol concentration, its effect being not only greater at each period but of longer duration Paredrine ranks second in effectiveness and adrenalin third Atropine seems to be the least effective of the four The question arises whether there is a fundamental difference in the action of these substances A number of experiments seem to affirm this. In experiments in which paredrine raised the systolic blood pressure to a level considerably higher than amphetamine sulfate blood-alcohol concentration was depressed much more by the latter drug

DISCUSSION

The main factors which account for the concentration of alcohol in the blood are absorption, diffusion into the tissues, oxidation in the tissues, and excretion through the lungs and through the kidneys

Excretion through the lungs and through the kidneys

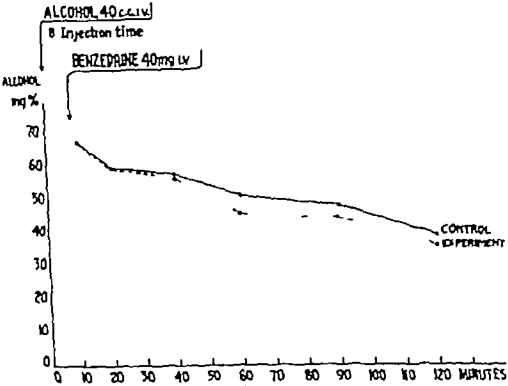
The loss of alcohol in the expired air and in the urine in the first two hours after the ingestion of alcohol is so small (6), (7), that even twice or thrice that amount could not possibly explain the marked depression of the alcohol concentration in the blood under the influence of the drugs used in these experiments Besides, we have never observed increased respiration in our patients following administration of amphetamine, and this is in conformity with the prevailing opinion in the literature We also found in 15 determinations that the alcohol excretion through the urine after drug administration never exceeded that found during the control period, except in the case of adrenalin, which reduced the urinary excretion of alcohol

Oxidation

The source of the greatest loss of alcohol in the body is oxidation according to Haggard and Greenberg (6) The rate of oxidation per hour is 17 6 per cent of the amount of alcohol present in the tissues Within 16 hours, 90 per cent of the alcohol is oxidized and only 10 per cent is excreted The question arises as to whether or not the drugs used increase the oxidation rate of alcohol No experimental data have as yet been published on the influence of amphetamine on the oxidation of alcohol According to the unpublished data of Stotz's careful investigations of tissues in the Warburg apparatus, amphetamine sulfate inhibits the oxygen consumption of rat brain oxidizing glucose and, even if given in rather large concentrations, has no effect on the oxidation of alcohol by liver tissue

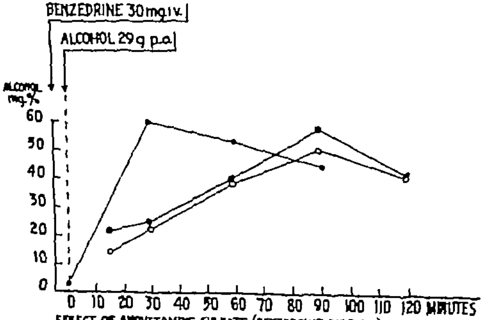
In our own experiments on the effect of amphetamine on the metabolic rate (12) (13), we found it to have no appreciable effect on the basal metabolism This is in agreement with the bulk of the work done by others, although there are some who found a moderate increase in basal metabolism

If amphetamine sulfate were to increase the oxidation of alcohol, it would be expected that the blood-alcohol curve following intravenous injection of alcohol would be considerably lower under the influence of this drug That this is not the case is shown in figure 6 which represents the results of two experiments As will be seen, the control and experimental curves are almost identical This is further shown by figure 8, which represents experiments in which amphetamine was administered at the time when the blood-alcohol curve following oral administration of alcohol had reached its peak, indicating the height of absorption The same result with regard to adrenalin



EFFECT OF AMPHETAMINE SULPHATE (BENZEDRINE) ON BLOOD ALCOHOL CURVE WHICH WAS OBTAINED BY INTRAVENOUS INFUSION OF ALCOHOL SOLUTION

Fig 6



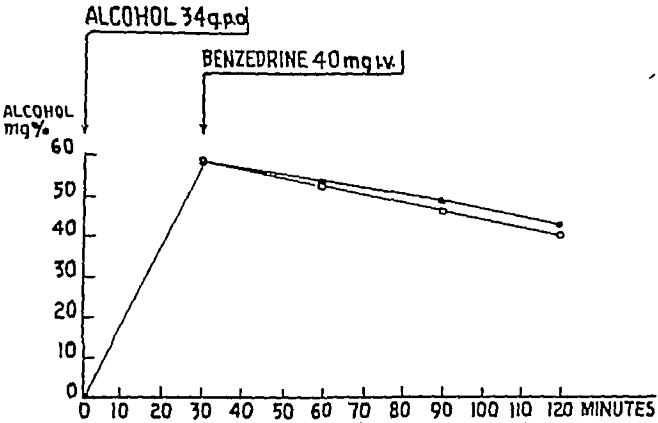
EFFECT OF AMPHETAMINE SULFATE (BENZEDRINE SULFATE) ON ALCOHOL CONCENTRATION IN ARTERIAL AND VENOUS BLOOD
CONTROL ARTERIAL BLOOD VENOUS BLOOD

Fig 7

was obtained by Fleming and Reynolds (1), who showed that adrenalin did not alter the blood-alcohol curve if alcohol was injected intravenously Thus, it may safely be stated that amphetamine does not increase the oxidation of alcohol

Diffusion

When alcohol is orally administered under normal conditions, during the period of absorption the alcohol concentration in the arterial blood is distinctly greater than that of the venous blood (6) This difference represents the amount which has diffused into the tissues We felt it necessary, therefore, to determine the alcohol concentration in the arterial as well as in the venous blood under the influence of amphetamine sulfate Blood samples



EFFECT OF AMPHETAMINE SULFATE (BENZEDRINE SULFATE) ON ALCOHOL CONCENTRATION IN VENOUS BLOOD WHEN INJECTED AT HEIGHT OF ALCOHOL CONCENTRATION •—• CONTROL o—o EXPERIMENT

FIG 8

were accordingly withdrawn within one minute of each other by puncture of the brachial artery and the basilic vein The result of such an experiment is represented in figure 7 It shows that the alcohol concentration of the arterial blood is low, just as is the alcohol content of the venous blood, the arterio-venous alcohol difference being lessened as compared to the control blood This indicates that whatever depression these drugs produce in the content of the venous blood must be due to decreased absorption of alcohol from the gastrointestinal tract

Absorption

The correctness of this conclusion is indicated by the following

1 The shape of the blood-alcohol curve following the oral and duodenal administration of the chemical substances The alcohol curve shows a

gradually ascending slope in which the peak has been shifted to the right as compared to the control curve

2 If the assumption is correct that these drugs delay the absorption of alcohol, then injection of them at a time when the absorption is nearly complete should not alter the blood-alcohol curve. That this is true is shown in figure 8 which represents an experiment in which 40 mgm. of amphetamine sulfate were injected at the time when the control curve was at its maximum. There was practically no change in the experimental curve, which is almost the same as the control.

3 In our own experiments (figure 6) and those of Fleming and Reynolds (1) the chemicals did not produce any appreciable alteration of the blood alcohol curve following direct intravenous injection of alcohol. (The small amounts of alcohol which still permeate into the gastrointestinal tract and thence are reabsorbed can safely be neglected.)

4 The question still remains whether a delay of the emptying time of the stomach may be a factor in producing the depressed blood-alcohol curves which we obtained in our experiments. We therefore in a few cases administered the alcohol by duodenal tube—a procedure which, for the same reason, was first carried out in dogs by Haggard and Greenberg (6) In contrast to the curves obtained when alcohol was given orally the peak of the blood-alcohol concentration was reached within five to twenty minutes when it was given duodenally When this same experiment was repeated a few days later, with the difference that amphetamine was injected about five minutes prior to the administration of the alcohol, the result was a definite diminution of the blood-alcohol concentration of from 56 1 to 80 per cent as compared to the control curve This effect, however was of shorter duration than when alcohol was given orally but had disappeared 30 to 40 minutes after the administration of the alcohol We conclude from these experiments that amphetamine sulfate delays the absorption of alcohol from the intestines, and that the emptying time of the stomach also plays an important rôle in the duration of the effect.

5 More direct evidence of inhibited absorption of alcohol under the influence of sympathomimetic drugs is presented by the work of Hanzlik and Collins (8) These authors, in quantitative studies on the gastrointestinal absorption of drugs in animals investigated the absorption of alcohol from intestinal loops which were previously treated with epinephrine While the average percentage of absorbed alcohol from the untreated loops was 55.2 from the treated loops it was only 2.89 per cent. The effect of epinephrine on absorption, however, is not limited to alcohol alone. We know from the work of Exner (9) Meltzer and Auer (10), in 1903 and 1904 that by the parenteral administration of adrenalin the absorption of orally administered substances, like strychnine and physostigmine, is also retarded.

Both on the basis of our own experimental data and the experiments which

we have cited from the literature, we ascribe the lowering of the blood-alcohol concentration to the interference by these drugs with the absorption of alcohol from the alimentary tract As to the probable basis of this inhibitory effect, Hanzlik and Collins (8) ascribe it to changes in the local gastrointestinal circulation by vasoconstriction, while Meltzer and Auer (10) discuss the possibility that these substances decrease the vital permeability of the capillary wall which brings about the retardation of absorption and transudation

SUMMARY AND CONCLUSIONS

1 Experiments are reported in which the effects of amphetamine (benzedrine) sulfate, β-parahydroxyphenylisopropylamine (paredrine), adrenalin chloride and atropine sulfate on the blood-alcohol concentration following the oral administration of alcohol were investigated

2 These drugs lower the blood concentration level of alcohol, probably by inhibiting the absorption from the alimentary tract and by delaying the emptying time of the stomach

3 Quantitatively, amphetamine sulfate is the most effective of this group It is followed in order of effectivity by paredrine, adrenalin chloride and atropine sulfate

We wish to acknowledge our indebtedness to Dr E Stotz, biochemist at the McLean Hospital, Waverley, Massachusetts, for permitting us to report the results of his experiments in this paper, details of which will be published by him in a separate paper

We also with to thank Miss Helen N Keane and Miss Mollie S Levin for their assistance in the preparation of this work

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THE POTENTIATION AND PARALYSIS OF ADRENERGIC EFFECTS BY ERGOTOXINE AND OTHER SUBSTANCES

CHANG-SHAW JANG

From the Pharmacological Laboratory College of the Pharmaceutical Society London

Received for publication July 30 1940

Since many of the sympathomimetic amines have a double action in relation to adrenaline and the adrenergic transmitter synergistic in low concentrations and antagonistic in high ones (1) it is of great interest to see if some of the well known adrenaline-antagonists or in Lapicque's terminology sympatholytics , may possibly act as synergists in low concentrations. Indeed this possibility has already been suggested by some 'atypical results scattered in the literature Bacq and Fredericq (2) found that F 933 one of the well known synthetic antagonists, in small doses increases the effect of sympathetic stimulation on the cat's nictitating membrane The effect of circulating sympathin on the heart is according to Cannon and Bacq (3), much increased after a small dose of ergotamine Very recently Herwick, Linegar and Koppanyi (4) found that small doses of ergotoxine increase the pressor effect of adrenaline in cats anaesthetized with barbiturates These observations are rather surprising but it surely would be wrong to dismiss them as "atypical cases

In the present investigation the sympatholytics used were ergotoxine yohimbine and F 933 the last one was kindly supplied by Prof Fourneau Experiments were done on the perfused rabbit's ear the frog's heart and the spinal cat. Methods are exactly the same as those already described (1)

RESULTS

(1) *F 933 (piperidylmethylbenzodioxane)* The effects of this drug on adrenergic transmission in the rabbit's ear are very interesting In suitable concentrations it increased both the effect of adrenaline and that of adrenergic stimulation on the rabbit's ear but the sensitizing concentration for the nervous effect was higher than that for the adrenaline effect Figure 1B shows that F 933 in a concentration of 10^{-8} definitely increased the effect of adrenaline but the effect of adrenergic stimulation was not altered after the drug was washed out by long perfusion with plain Locke's solution and the effects of adrenaline and nervous stimulation had become steady (C) reperfusion with F 933 in a higher concentration (5×10^{-8}) then increased

the nervous effect but decreased the adrenaline effect, (D) Still higher concentrations up to 10^{-5} greatly diminished or completely suppressed the adrenaline effect but left that of adrenergic stimulation intact or only slightly

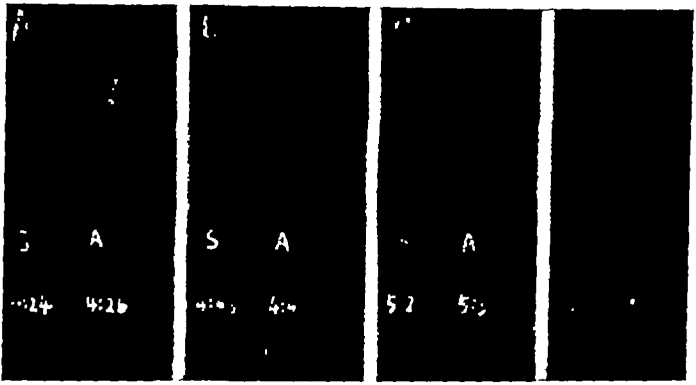


FIG 1 OUTFLOW FROM THE PERFUSED RABBIT'S EAR

The height of this and similar records is proportional to the time interval between successive drops *S* sympathetic stimulation 2 sec, *A*, 0 1 cc adrenaline, 2×10^{-7} (*A*) Perfusion with Locke's solution (*B*) F 933 10^{-6}, started at 4 30 (*C*) Locke's solution at 4 50 (*D*) F 933 5×10^{-6} at 5 33

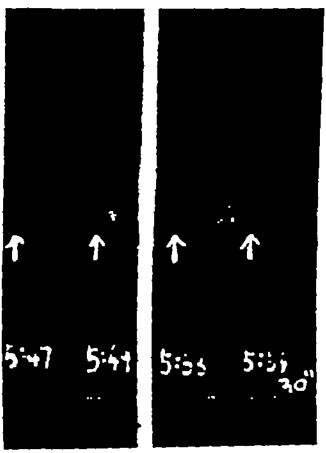


FIG 2 RABBIT'S EAR PERFUSION

Effects of equal doses of adrenaline (0 1 cc, 2×10^{-7}) before and after perfusion with yohimbine (10^{-6}), started at 5 50

diminished, thus confirming Wierzuchowski's result on the perfused rabbit's head (5) In concentrations of 10^{-4} or above, it also markedly diminished the nervous effect

F 933 moderately excited the frog's heart in concentrations varying from 2×10^{-6} to 2×10^{-5} and depressed it in higher ones, confirming Shen's result (6). But contrary to his observation that F 933 in a concentration of 10^{-4} reversed the effect of adrenaline (3×10^{-6} to 2×10^{-5}), I found that the effect of adrenaline in concentrations between 10^{-5} and 10^{-4} was only reduced or suppressed. In low concentrations (10^{-8} to 10^{-9}) F 933 occasionally sensitized the heart to the action of adrenaline but this sensitization was never marked.

(2) *Yohimbine* In very low concentrations yohimbine also was able to increase the effect of adrenaline in the perfused rabbit's ear (fig 2). Its influence on the adrenergic stimulation in such concentrations was very variable. No definite effect was observed on the action of adrenaline on the frog's heart with yohimbine in concentrations below 10^{-5} at which however,

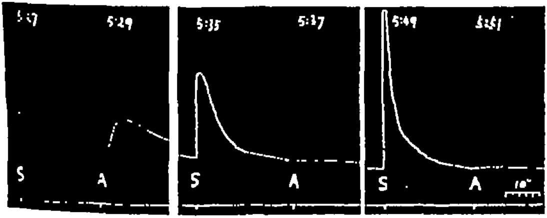


Fig 3. Cat's Nictitating Membrane

S sympathetic stimulation coil 6 3 sec *A* 4 μg. adrenaline intravenous 3 mgm. yohimbine given at 5 33

yohimbine slightly depressed the heart and markedly diminished but never reversed the adrenaline action

In spinal cats, an ordinary dose (about 1 mgm. per kilogram) reversed the pressor effect of adrenaline and completely suppressed its effect on the nictitating membrane but the effect of sympathetic stimulation on the membrane was only slightly diminished at first and became much larger after a while (fig. 3) A few attempts to get sensitization with smaller doses were not successful

(3) *Ergotoxine* In a cat given dial and cocaine Gaddum and Goodwin (7) got the same result as Herwick Linegar and Koppanyi (4), who reported that an ordinary dose of ergotoxine increases the pressor effect of adrenaline instead of reversing it. In the spinal cat however very small doses (0 1 mgm. per animal for instance) antagonized the effect of adrenaline on the nictitating membrane and on the blood pressure Figure 4 shows the effect of sympathetic stimulation and of intra-arterial injection of adrenaline on the nictitating membrane and blood pressure Before ergotoxine was given

both procedures produced good effects on the membrane but none on the blood pressure, after ergotoxine the effect of nervous stimulation on the mem-

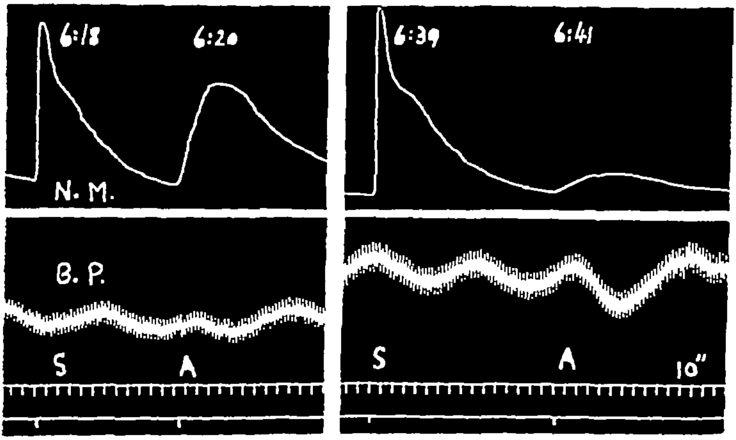


FIG 4 SPINAL CAT

Upper tracing, contractions of nictitating membrane, lower, blood pressure S, sympathetic stimulation, coil 8, 3 sec A, 2 μg adrenaline, intra arterial Ergotoxine, 0 1 mgm , in three divided doses given intravenously at 6 22 6 27 and 6 32

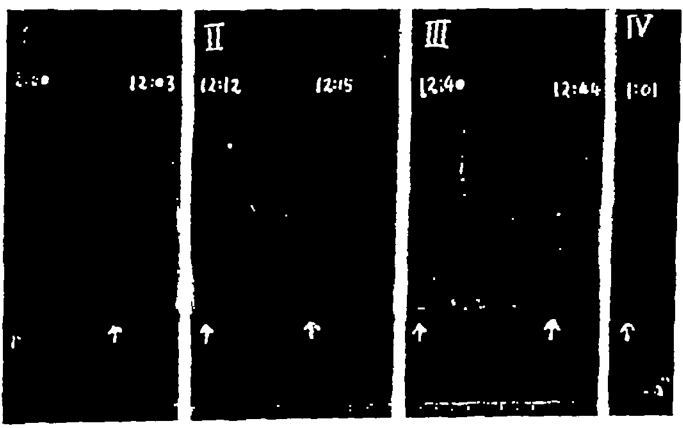


FIG 5 RABBIT'S EAR

Effects of equal doses of adrenaline (0 1 cc , 10^{-7}) during perfusion with (I) Locke's solution, (II) ergotoxine 10^{-6}, started at 12 08, (III) Locke s solution at 12 20, and (IV) ergotoxine 10^{-6}, at 12 50

brane was greater but that of adrenaline was almost completely suppressed, the hitherto unaffected blood pressure now fell with the same intraärterial dose of adrenaline

On the rabbit's ear, ergotoxine in very low concentrations (10^{-9} to 10^{-10}) increased the effect of adrenaline and this sensitization was readily reversible, at slightly higher concentrations, however, the antagonistic effect began to dominate, but the actual reversal of the adrenaline action was not observed even in high concentrations (fig 5)

DISCUSSION

Sympathomimetics and their synergists and antagonists

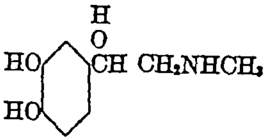
It has long been suspected that synergists and antagonists to adrenaline might be themselves sympathomimetic compounds. Bacq (8) indeed, compiled two tables in which various actions of ergotoxine and ergotamine are compared side by side with those of adrenaline on different organs *in vitro* and *in vivo* in order to show that the pharmacological properties of these ergot alkaloids are in many respects actually quite similar to those of adrenaline He believes that these alkaloids antagonize the adrenaline action because they modify the physico-chemical properties of cells in the same manner as adrenaline does. Yohimbine too, has many actions in common with those of adrenaline (9) Cocaine, besides its proper status as a local anaesthetic, has also been classified among the "sympathetic stimulants because it increases the sympathetic excitability' (10) Recently the sympathomimetic nature of cocaine has been further clarified (11)

With the appearance of many synthetic adrenaline antagonists it has become clear that a slight change in chemical structure can transform the sympathomimetic amines into their antagonists. There are also compounds that can be both sympathomimetic and sympatholytic

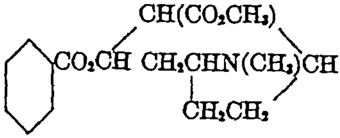
The results of the present and the previous investigations (1) revealed that many of these sympathomimetic amines can be both synergistic to adrenaline like cocaine and antagonistic like ergotoxine Moreover mere variation in concentration can transform cocaine, the well known synergist, into an antagonist, and can transform ergotoxine yohimbine and F 933, the well known antagonists, into synergists. It is therefore quite clear that the capacity to potentiate or to antagonize the action of adrenaline can no longer be considered specific for the synergists and antagonists investigated.

From the chemical point of view adrenaline, ergotoxine and cocaine, each representing one of the three groups, appear quite different from one to another Even so it is not impossible to find some significant chemical similarity among these apparently heterogenous compounds. Although much work has been done on the correlation of chemical structure with physiological activity of sympathomimetic amines (12) since the publication of Barger and Dale s classical work on this subject, nothing much has been added to their conclusions that the aliphatic amine group is essential for the characteristic sympathomimetic properties, and that an approximation to adrenaline in structure is attended with increasing specificity of the action

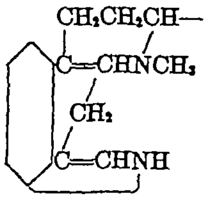
Now let us examine the chemical structure of adrenaline, cocaine and ergotoxine



Adrenaline



Cocaine



Ergotoxine (represented by the tentative structural formula of the part of lysergic acid, which is supposed to be the most essential part of ergotoxine)

The structural similarity of these three compounds, so far as the essential structure for sympathomimetic activity is concerned, is striking enough All of them possess one or two side chains of aliphatic amines The structures of cocaine and ergotoxine, of course, are not represented in the orthodox way, but that is immaterial The closing up of the straight chains with the formation of rings does certainly modify much of the sympathomimetic activity, but does not necessarily abolish it (13)

The structural similarity between the synthetic sympatholytics and the sympathomimetics is still more striking, and in some cases one can hardly tell any difference between them For instance, among the three isomers of *1*-oxyphenoxy-*2*-methylamino-ethane, the *meta* compound is a sympathomimetic, the *ortho* compound a sympatholytic, and the *para* both sympathomimetic and sympatholytic (14)

It has been suggested that the phenoxy group of these synthetic sympatholytics is essential for the antagonistic activity (15) This is not true, because in the first place many compounds such as ergotoxine and yohimbine do not possess this group and yet its absence does not prevent their being strong antagonists, in the second place, many of the typical sympathomimetic amines themselves can act as antagonists under certain conditions (1), and finally, as has just been discussed in the previous paragraph, some of the

phenoxyethylamines are themselves sympathomimetics in spite of the presence of this "sympatholytic group"

From my results there is little doubt that apart from the group which is essential for sympathomimetic action, there is actually no chemical group in all these drugs which can be considered specific for the synergistic or antagonistic property towards adrenaline On the other hand, various groups such as the phenoxy, and various structural changes such as the closing up of the side chain, which tend to cut down the sympathomimetic activity, seem only to disclose the inherent synergistic or antagonistic properties, which are normally more or less masked by the strong sympathomimetic properties. The apparent absence of synergistic and antagonistic properties in nor adrenaline and epinine (1) is perhaps due to the fact that their sympathomimetic activity is too close to, and as specific as that of adrenaline It may be assumed that it is the same chemical group which is responsible for the synergistic and the antagonistic as well as for the sympathomimetic properties in all these drugs we have studied, and this very group is the aliphatic amine.

Dissociation of the effect of adrenergic stimulation from that of adrenaline

The ability of Fourneau's dioxane compounds to dissociate the effect of adrenergic stimulation from that of adrenaline has been extensively investigated by previous workers The present investigation has revealed that in addition to F 933 both yohimbine and ergotoxine possess this dissociating power, i e., in concentrations or doses sufficient to abolish the effect of adrenaline they have practically no effect on that of nervous stimulation or, as on the cat's nictitating membrane, they even increase it. Further, not only for antagonism but also for sensitization a higher concentration of F 933 is required to modify the nervous effect than to modify the adrenaline effect. The fact that even some of the general anaesthetics possess this dissociating action (16) indicates its non-specificity

Various theories have been proposed to explain the relative ease with which the response to adrenaline is depressed by F 933 Melville (17) believed that the facts can be explained by supposing that the substance liberated by adrenergic nerves is not adrenaline but noradrenaline This explanation cannot be applied to the observations on the rabbit's ear since it has been shown that the substance liberated by these nerves is not noradrenaline (18)

Bacq and Monnier (19) concluded that chemical transmission is not the only mechanism by which impulses can pass to the effector cells and that when the chemical transmitter has been antagonized some other mechanism, presumably electrical remains effective.

Cannon and Rosenblueth (20) have adopted a theory, which was proposed by Dale and Gaddum (21) to explain similar anomalies in the interaction of atropine and acetylcholine on cholinergic transmission and have concluded that adrenaline introduced by the blood stream and circulating sympathin are more easily antagonized because they are barred from access to the re-

ceptors by the action of the antagonists on some membranes, while the chemical transmitter liberated locally is in more direct contact with the receptors behind the membrane

My results speak more in favor of the membrane theory than of the dual transmission theory of Bacq and Monnier The fact that higher concentrations of F 933 are required for both sensitization and antagonism of the nervous effect than of the adrenaline effect suggests that the dissociation of these two effects is due to their spatial rather than to their qualitative difference

SUMMARY

1 Ergotoxine, yohimbine and F 933 were examined on the rabbit's ear, the frog's heart and the spinal cat for their interaction on adrenergic transmission

2 In very low concentrations these substances are all capable of sensitizing the rabbit's ear to the action of adrenaline No definite sensitization occurs in the other two preparations In higher concentrations they antagonize the effect of adrenaline and adrenergic stimulation

3 On the cat's nictitating membrane all three substances can abolish the effect of adrenaline without diminishing the effect of sympathetic stimulation Indeed this latter effect may be increased

4 It is pointed out that the action of ergotoxine tow[illegible]naline is fundamentally similar to that of cocaine

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ADRENALINE DESTRUCTION IN THE LIVER AND METHYLENE BLUE

FLORA J PHILPOT AND G CANTONI

From the Laboratories of Biochemistry and Pharmacology Oxford

Received for publication August 9 1940

The part played by the liver in the destruction of adrenaline in the body has been studied by several workers. Elliott (1) quotes the work of Battelli (2), who perfused blood containing adrenaline through the liver and observed the adrenaline to disappear at a rate that varied with the oxygen consumption, he also quotes the work of Langlois (3), who found that slow injection of adrenaline into the mesenteric veins failed to cause a rise of blood pressure. Having repeated these observations but having also observed that adrenaline is inactivated when perfused through many different organs, Elliott concluded that the liver did not play a major rôle.

More recently Markowitz and Mann (4) compared the action of adrenaline before and after hepatectomy in the dog and found that the duration of the pressor action was not significantly increased by this operation They confirmed the observation that the pressor action of adrenaline injected into the mesenteric vein was much less than that of the same dose injected into the jugular vein. Since they found that the pressor action of adrenaline injected into the femoral artery was also much less than that of adrenaline injected into the femoral vein they concluded that the smaller effect after mesenteric injection and after injection into the femoral artery was due to the greater dilution in which adrenaline reached the general circulation These observations were confirmed by Gollwitzer Meier (5) and by Yoshi yuki (6) who showed that the dose injected into the mesenteric vein must be five times as great to produce the same effect as in the jugular vein this difference he found disappeared after hepatectomy Bacq (7) also confirmed these observations quantitatively and qualitatively but did not consider that there was specific destruction of adrenaline in the liver because of the similar difference between intraarterial and intravenous injection

The biochemical processes which may be involved in the oxidation of adrenaline in the body have recently been discussed by one of us (8) In order to throw more light on the problem a further study has been made of the oxidation of adrenaline by liver and by heart muscle in the presence of methylene blue *o*-cresolindophenol and potassium cyanide. The effect of methylene blue was investigated because it was found (Philpot (9)) to in-

hibit the action of amine oxidase, but not to affect that of other oxidases or dehydrogenases O-cresolindophenol was used as a control, that is to say as a redoxindicator which does not inhibit amine oxidase Potassium cyanide was used because it is known to inhibit the oxidation of adrenaline in the ring by cytochrome or catechol oxidases The results of this investigation, for which one of us (F J P) is responsible, are described in Part I They were clear enough to suggest a further physiological analysis which is described in Part II

PART I

Methods

1 Guinea-pig liver was ground in a mortar, squeezed through muslin and suspended in 0 01 M phosphate buffer, pH 7 0 This preparation was then shaken in air at 38° for 2 hours If this preliminary incubation was omitted it was observed that when adrenaline was added, and an attempt made to recover it immediately, the proportion recovered was very small If the attempt to recover it was made after 30–60 minutes the proportion recovered was larger The improvement in recovery was possibly connected with autolysis since in experiments with potassium cyanide, which catalyses autolysis, the initial recovery of adrenaline was higher, and there was no subsequent increase

2 Heart muscle was minced, ground with sand, squeezed through muslin, washed twice on the centrifuge, suspended in phosphate buffer 0 01 M, pH 7 0, and incubated at 38° for 30 minutes

A few experiments were done to follow the oxygen uptake manometrically, but in most of the work adrenaline was added to the suspension to give an initial concentration of 10^{-4}, and the suspension was shaken vigorously in air at 38° Samples were withdrawn at intervals and the residual adrenaline estimated by Shaw's (10) method modified as follows A 1 cc sample was added, with shaking, to 2 cc of 5 per cent trichlor acetic acid in a centrifuge tube After 2 minutes centrifuging the supernatant fluid was poured through a small wet filter paper, the precipitate washed with 1 cc water and the washing was filtered To the filtrate was added 0 8 cc 2 N sodium acetate This buffered the solution to about pH 4 6 The first adsorption with $Al(OH)_3$ was now carried out To the supernatant fluid was added 1 cc triethanolamine buffer, pH 8 3 (100 cc 4 9 N triethanolamine, 12 2 cc 10 N H_2SO_4, final volume 163 cc) The solution was again treated with $Al(OH)_3$ and the precipitate washed with the triethanolamine buffer diluted 1 in 100 The rest of the technique followed Shaw's method exactly When the liver suspension was previously incubated a fairly constant recovery of 85 per cent of added adrenaline was obtained By his technique Shaw claims 75 to 80 per cent recovery, but using his method exactly I obtained very erratic results

Samples were withdrawn immediately after adding adrenaline, and after 30 and 60 minutes incubation The control consisted of liver suspension with all additions except adrenaline

The following solutions were used Sodium acetate, 2 N Phosphate buffer, 0 01 N, pH 7 0 Adrenaline 1 in 10^4, dissolved in H_2SO_4 $\frac{N}{100}$, Triethanolamine, 4 9 N, H_2SO_4, 10 N, KCN, 0 1 M, Methylene blue, $\frac{M}{500}$, o-cresolindophenol, $\frac{M}{500}$, Cytochrome c, 2 65 × 10^{-4} M p-phenylenediamine-HCl, 0.2 M Other solutions as in Shaw (10)

Experimental results

1 Liver The rate of disappearance of adrenaline from a liver suspension is greatly reduced by methylene blue and is not affected by *o*-cresolindophenol KCN also inhibits to some extent. The inhibition by methylene blue and KCN add up to about 100 per cent. The oxygen uptake of liver suspension in the presence of *p*-phenylenediamine and cytochrome *c* is unaffected by methylene blue and *o*-cresolindophenol

TABLE 1

TISSUE	INHIBITOR	SUBSTRATE	CONCENTRATION	PER CENT INHIBITION
Liver	Methylene blue $\frac{M}{10\,000}$	Adrenaline	10^{-4}	60–80
Liver	*O*-cresolindophenol $\frac{M}{10\,000}$	Adrenaline	10^{-4}	0–10
Liver	KCN $\frac{M}{100}$	Adrenaline	10^{-4}	30
Liver	Methylene blue	Tyramine	$6\ 6 \times 10^{-4}$	80
Liver	*O*-cresolindophenol	Tyramine	$6\ 6 \times 10^{-4}$	0

The following volumes were used liver suspension 10 cc methylene blue and *o*-cresolindophenol 0.5 cc. adrenaline 0 1 cc KCN 1 0 cc.

These two results were obtained manometrically during previous work see Philpot (9)

TABLE 2

TISSUE	INHIBITOR	SUBSTRATE	CONCENTRATION	PER CENT INHIBITION
Heart (rabbit)	Methylene blue $\frac{M}{10,000}$	Adrenaline	5×10^{-4}	0
Heart (pigeon)	Methylene blue $\frac{M}{10,000}$	Adrenaline	10^{-4}	14
Heart (dog)	Methylene blue $\frac{M}{10\,000}$	Adrenaline	10^{-4}	16–20
Heart (dog)	KCN $\frac{M}{100}$	Adrenaline	5×10^{-4}	100

The following volumes were used heart suspension 10 cc methylene blue 0.5 cc. adrenaline 0 1 cc KCN 1 cc

2 Heart muscle The oxygen uptake of a preparation of ground rabbit heart muscle in the presence of adrenaline (initial concentration 5×10^{-4}) and cytochrome *c* is unaffected by methylene blue Dog heart muscle was treated as described above The rate of disappearance of adrenaline (initial concentration 10^{-4}) from this suspension was only slightly affected by methylene blue.

Principal conclusions 1 Methylene blue inhibits the oxidation of adrenaline by the liver but *o*-cresolindophenol does not Potassium cyanide has

some inhibitory effect, but much less than methylene blue The "methylene blue-sensitive" system and the "cyanide-sensitive" system together account for all the destruction of adrenaline by the liver

2 Methylene blue has little or no inhibitory effect on the oxidation of adrenaline by heart muscle, whereas potassium cyanide has a powerful inhibitory action

PART II

Splenic and jugular injections A further study has been made by one of us (G C) of the difference in the pressor response to adrenaline injected into the jugular vein and into the splenic vein Adrenaline has been compared

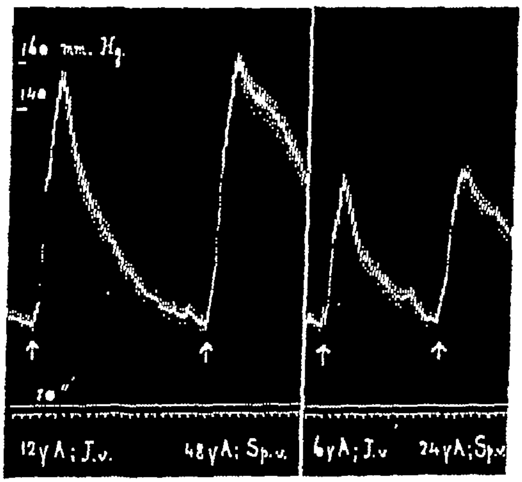

FIG 1 SPINAL CAT, BLOOD PRESSURE

Equipressor doses of adrenaline injected into the jugular vein and into the splenic vein

with other substances such as tyramine, corbasil, and notably with pituitary (posterior lobe) extract The effect of methylene blue and of *o*-cresolindophenol has been examined The experiments have been carried out in the spinal cat prepared by Dale's method (11) in which the cord is cut at the level of the second cervical vertebra Injections were made into the jugular vein, and also into a cannula tied into the splenic vein near its junction with the portal vein, so that solutions could be injected into the portal stream without interrupting the intestinal circulation The vessels entering and leaving the spleen were tied in three ligatures

The record in figure 1 confirms the finding that the dose of adrenaline injected into the splenic vein must be much bigger than the dose injected into

the jugular vein to have the same pressor effect. Thus in figure 1 0 048 mgm. injected into the splenic vein was similar in effect to 0 012 mgm injected into the jugular vein The second part of figure 1 shows that the same relation held when the doses were halved

Not only adrenaline, but also tyramine and corbasil were examined in this way As shown in table 3 the average ratio for adrenaline was 4 7 that is to say in seven experiments it was found that the dose injected into the splenic vein had to be an average of 4 7 times the dose injected into the jugular to obtain the same pressor effect For tyramine the average figure was similar namely 4 6 For corbasil however the average figure was less namely 2 6 This suggested that the proportion of corbasil destroyed in the liver was less than that of tyramine or adrenaline as would be expected from the fact that its molecule contains a $-CH_3$ group attached to the α carbon atom, which prevents its oxidation by amino oxidase

TABLE 3

Ratio of equipressor doses injected in splenic and jugular veins

EXPERIMENT	ADRENALINE	TYRAMINE	CORBASIL	PITUITARY EXTRACT
1	4			1
2	10			1 2
3	4	6		1
4	3 3	3 3	2	
5	5	5	3 5	
6	3 3		2 3	
7	8 2	4		
Mean	4 7	4 6	2 6	1

The most interesting result however was that obtained with pituitary (posterior lobe) extract. For this as illustrated in figure 2 the ratio was 1 There was no significant difference between the effect of a dose injected into the jugular and into the splenic vein By both routes the pressor effect was the same

The action of methylene blue The action of methylene blue and o-cresolindophenol has been tested as shown in figure 3 At A is shown the effect of injecting 3 0 cc. adrenaline 10^{-6} into the splenic vein at a uniform rate during 45 sec. At B this injection was repeated. At C the same amount of adrenaline was injected in a solution of o-cresolindophenol of strength $\frac{m}{5000}$ The pressor action of adrenaline was the same though slightly prolonged. At D the adrenaline was injected in a solution of methylene blue also $\frac{m}{5000}$ As figure 3 shows the pressor action of the adrenaline was greatly increased It

should be stated that this amount of methylene blue injected alone into the splenic vein had no pressor effect

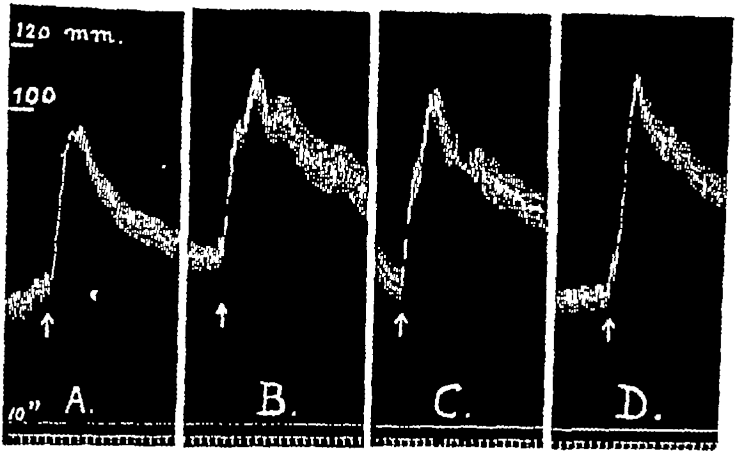

FIG 2 SPINAL CAT, BLOOD PRESSURE
Effects of 0 2 unit pituitary (post lobe) extract injected into the jugular vein (A and D), and into the splenic vein (B and C)

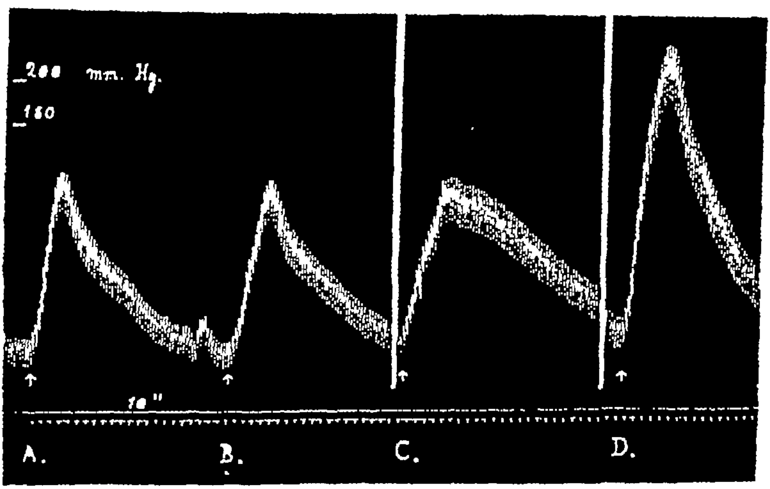

FIG 3 SPINAL CAT, BLOOD PRESSURE
Effect of 3 cc adrenaline 10^{-6} (A and B), 3 cc adrenaline 10^{-6} in *o* cresolindophenol $\frac{m}{5000}$ (C), 3 cc adrenaline 10^{-6} in methylene blue $\frac{m}{5000}$ (D) Each injection took 45 secs

The effect of methylene blue in augmenting the pressor action of adrenaline was next examined when injections were made into the jugular as well as

into the splenic vein It was found that injections into the jugular vein were also augmented, but not so much, so that the ratio of equipressor doses of adrenaline was reduced

Table 4 summarizes the results of three experiments which show that after the injection of $\frac{m}{500}$ methylene blue into the jugular vein in such a dose as to give a concentration in the blood of approximately $\frac{m}{10,000}$, the dose of adrenaline which injected into the splenic vein gave the same rise of blood pressure as a dose injected into the jugular vein was no longer 4 6 times, but only twice the latter

Methylene blue augmented the pressor action of adrenaline injected into the jugular vein by an average figure of 60 per cent that is to say the rise produced by a given dose after administration of methylene blue was equivalent to that produced by a 60 per cent greater dose given before. This result

TABLE 4

Ratio of equipressor doses of adrenaline injected into the splenic and jugular veins

EXPERIMENT	BEFORE INJECTION OF METHYLENE BLUE	AFTER INJECTION OF METHYLENE BLUE
8	4	2
9	6 6	2 5
10	3 2	1 5
Mean	4 6	2

was taken from 15 experiments On the other hand methylene blue augmented the pressor action of adrenaline injected into the splenic vein by 300 per cent The pressor action of adrenaline was not augmented by *o*-cresolindophenol at all.

Methylene blue on the isolated heart. Some experiments have been carried out to discover whether methylene blue augmented the action of adrenaline on the isolated heart of the cat perfused with oxygenated Locke-Ringer solution by Langendorff's method no augmentation was observed in the amplitude of the contractions such as is readily seen when cocaine is used (Burn and Tainter (12)) Methylene blue as already observed by Sachs (13) exerted a depressant effect on the heart muscle in higher concentrations

DISCUSSION

It has been shown on the one hand that when adrenaline is destroyed *in vitro* in the presence of liver suspension the rate of destruction is greatly diminished by introducing methylene blue. It is known that this substance inhibits the action of amine oxidase *O*-cresolindophenol on the other hand, which does not inhibit the action of amine oxidase has no effect on the rate

of destruction of adrenaline in the same conditions. These results support the view that in the liver the destruction of adrenaline can be effected by amine oxidase

Experiments *in vivo* have been carried out which indicate that in fact this is the main mechanism of adrenaline destruction at work in the liver When adrenaline is injected into the splenic vein and carried to the portal system it exerts a pressor action equal to that produced by about one quarter of the dose injected into the jugular vein The small effect of injection into the portal system has been ascribed by several workers to the greater dilution which the adrenaline undergoes in the liver, but it has now been found that no similar difference exists for pituitary (posterior lobe) extract The explanation that adrenaline injected into the splenic vein loses the greater part of its pressor action because of dilution is therefore no longer tenable

It has also been found that the pressor effect of adrenaline injected into the splenic vein is much increased by the simultaneous injection of methylene blue, but is unaffected by the simultaneous injection of o-cresolindophenol The pressor effect of adrenaline injected into the jugular vein is also increased by methylene blue, but this increase is much smaller than that seen when the adrenaline goes directly to the portal system

These results lead us to disagree with Richter and Tingey (14) who doubt whether amine oxidase is involved in the normal destruction of adrenaline at all We think that in the liver it is the principal mechanism The part played by amine oxidase in other tissues of the body is difficult to establish In the animal from which the liver has been excluded by evisceration the injection of methylene blue sometimes increases the pressor action of adrenaline but often it does not In heart muscle methylene blue inhibited the destruction of adrenaline *in vitro* by less than 20 per cent, *in vivo*, however, methylene blue did not appreciably affect the action of adrenaline on the isolated perfused heart As has been suggested by Philpot (8) it is possible that cytochrome oxidase plays a part in the oxidation of adrenaline Heart muscle is extremely rich in this enzyme and *in vitro* the oxidation of adrenaline in heart muscle is greatly inhibited by KCN Unfortunately this experiment cannot be repeated *in vivo* owing to the essential rôle played by cytochrome oxidase in the respiration of all tissues The fact that the inhibition, by methylene blue and by KCN, of the destruction of adrenaline by liver add up roughly to 100 per cent, suggests that even in liver both types of oxidation may occur and that the relative amount of each enzyme present decides which type of oxidation takes place It is possibly significant that those tissues richest in cytochrome oxidase are poorest in amine oxidase

Since this work was completed, Richter (15) has published a preliminary note saying that esterification and not oxidation is the main fate of adrenaline administered by mouth We do not consider that this conclusion, if true, in any way conflicts with our results

SUMMARY

1 The destruction of adrenaline *in vitro* by a liver suspension is inhibited by methylene blue but not by o-cresolindophenol The former dye inhibits amine oxidase whereas the latter does not.

2 Adrenaline injected into the portal circulation has less pressor action than when injected into the jugular vein To obtain equal effects by the two routes the portal dose must be 4–5 times as great as the jugular dose No such difference exists for pituitary (posterior lobe) extract

3 The pressor effect of adrenaline injected into the portal system is greatly augmented if given simultaneously with methylene blue but not if given simultaneously with o-cresolindophenol.

4 The pressor effect of adrenaline injected into the jugular vein is augmented after the injection of methylene blue but much less than that of adrenaline injected into the portal system.

5 The evidence indicates that in the liver *in vivo* the main instrument of adrenaline destruction is amine oxidase.

The authors wish to express their thanks to Prof J H. Burn for his constant advice and assistance and to Dr E Bülbring for assistance with some of the experiments.

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EXPERIENCES WITH THE BIOLOGICAL ASSAY OF SEVERAL SYMPATHICOTONIC SUBSTANCES INCLUDING EPINEPHRINE

AXEL M. HJORT, EDWIN J. DEBEER AND LOWELL O. RANDALL

From The Burroughs Wellcome & Co. U. S. A. Experimental Research Laboratories, Tuckahoe, N. Y.

Received for publication July 2, 1940

While investigating the relative circulatory effects of some phenethylamine derivatives we found that the often used procedure wherein epinephrine, in an arbitrarily chosen dose, is used as the reference standard, was not quite adequate for our purpose. For example, a dose of 530×10^{-7} mM. per kilogram of *3,4*-dihydroxyphenethylmethylamine caused the dogs' blood pressure to rise, on the average, 30 mm. Hg. For the same response 46×10^{-7} mM. per kilogram of epinephrine was necessary, which means, that the latter was 11.5 times as potent a pressor substance as the former. But if the quantities of both of these compounds were determined for a response of 100 mm. Hg it was found that 312×10^{-7} mM. per kilogram of epinephrine was equivalent to 2600 mM. per kilogram of *3,4*-dihydroxphenethylmethyl amine or that epinephrine was now but 8.3 times as potent as the other amine. This discrepancy is due to a fundamental difference in the two dose-response curves. Also unless a large amount of data is collected errors due to biological variations may further modify the figures. Therefore, it becomes necessary to obtain the characteristics of the dose-response curves by some means such as the administration of graded doses and then, if the comparison is permissible, to use a procedure designed to reduce the error.

On examination of the literature a number of different procedures were found which have been proposed for the biological assay of epinephrine. Elliott (1), employing vagotomized cats anesthetized with urethane, estimated epinephrine quantitatively by the intravenous injection of increasing doses and interpolation from the curve drawn through the blood pressure maxima. He claimed that a perfectly uniform reaction to epinephrine was obtained. In a later publication (2) he had modified his procedure to the extent of destroying the brains and the cords (down to the 4th thoracic segment) of cats anesthetized with ether and stated that the blood pressures of such animals responded to any dose of epinephrine with the accuracy of a chemical balance. In 1924 Trendelenburg (3) felt that the Elliott (2) procedure was superior to others for the biological assay of epinephrine

Lyon (4), employing essentially the second Elliott procedure, found that the blood pressure response was conditioned by its level just prior to the injection of epinephrine Wilkie (5) introduced an improvement in the second Elliott procedure by ligating the suprarenal veins in addition to pithing the brains and cords Finally, Rosenblueth (6) suggested a further improvement by adding sympathectomy

The foregoing is a brief résumé of methods which differ from the U S P XI procedure Rosenblueth (6) further suggested the employment of the nictitating membrane for the same purpose but since we were primarily interested in the effect of epinephrine and other amines on the general circulation we felt that a technique which recorded blood pressure changes would best serve our purpose

Careful analysis of data recorded in the publications quoted above, impressed us with the fact that no method was as accurate as claimed by Elliott (2) Therefore, we decided to use a procedure combining certain features of the increasing dose and of the U S P XI methods

EXPERIMENTAL

The U S P XI procedure was followed up to the point where epinephrine injections began At that juncture epinephrine was given in progressively increasing doses instead of determining the dose at which 30 to 60 mm Hg responses occurred Dogs of 8 to 13 kgm body weight were selected Dial, about 70 mgm per kilogram, intraperitoneally, served as the anesthetic The dosage increment was accomplished by increasing concentration, keeping the volume unchanged, 0 08 cc per kilogram The data analyzed below were obtained from 12 dogs

RESULTS

Comments on results

When very small doses of epinephrine are administered an early circulatory depressor phase occurs in most animals This phase very probably influences the height to which the initial pressor effect will go Furthermore, one cannot say that this depressor influence is absent when the response measured is purely pressor It may play an important rôle in limiting the absolute value of the method, not only because it is probably always in effect but because it may vary quantitatively between animals

After large doses of epinephrine, a secondary depressor effect follows the primary pressor action quite consistently but variably Therefore we avoided the very large doses used by Lyon (4) and Wilkie (5)

In our experience, although the data are limited, blood pressure responses to increasing doses of epinephrine are uninfluenced by sex, by the level of the resting blood pressure or by the relative initial sensitivity to that substance

Relative to the accuracy of our procedure the results with paired doses indicate that it compares favorably with published data of other increasing dose methods

Value of results

The epinephrine dose-response curve was studied by pooling all of the data so as to get the complete picture, examining by means of graphs and testing by standard methods of curve fitting as described by Snedecor (7) The latter treatment serves to test the validity of the observer's impressions The results summarized in table 1 are as follows

1 The relationship between dose and response is represented by a curved line which resembles a section of a parabola (fig 1) This curve appears similar to those published by Elliott (2) Lyon (4) and Rosenblueth (6) Some sections of this line curve so gradually that, for all practical purposes they may be considered straight. This holds true for that portion limited by doses of 55×10^{-7} to 293×10^{-7} mM per kilogram inclusive, and of the section specified by the U.S.P.XI in which the response must lie between 30 and 60 mm Hg

2 As figure 2 shows, the relationship between the log dose and the response is represented by a curve which consists of two intersecting straight lines. Each of these lines could be said to obey the Weber Fechner law which states that the response is proportional to the logarithm of the dose However, no single expression of the Weber Fechner law applies throughout the whole range of data

3 The curve which can be most easily applied to a set of data, either by inspection or by calculation is the straight line. As the foregoing and figure 3 show such a line can justifiably be fitted throughout the entire range of data only when log dose is plotted against log response. When any compound is examined for similarity to epinephrine with respect to circulatory pressor action it should be tested by a method of this kind If the slope differs from that characteristic of epinephrine, i.e , if the plotted line is not parallel to that plotted for epinephrine, the potency of the compound cannot be expressed in terms of fractions or multiples of the potency of epinephrine when this is taken as unity, since it will vary according to the response level at which comparison is made

Analysis of the results according to the foregoing involves the accumulation of a relatively large quantity of data When working with individual animals the data obtainable are relatively few and another error due to individual variation becomes evident These data for two animals are presented graphically in figure 4 The slope of the dose-response curve for one is considerably steeper than that for the other We consider the former of these two dogs relatively sensitive to epinephrine. Proper planning of the experiment would help to reduce the error thus introduced, i.e., by means of

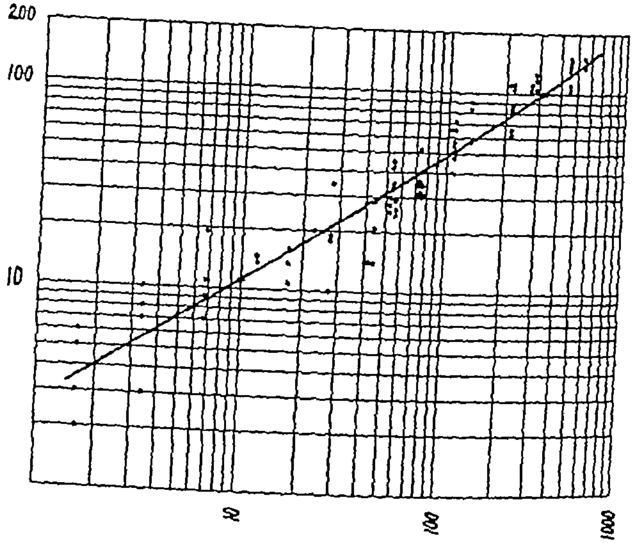

FIG 3 LOGARITHMIC DOSE-RESPONSE LINE
Abscissa is the logarithm of the dose Ordinate is the logarithm of the response

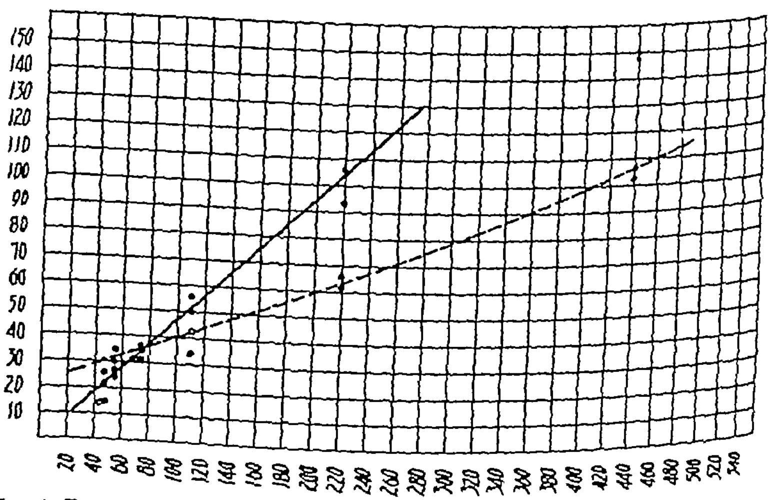

FIG 4 DIFFERENCES IN SLOPE OF ARITHMETIC DOSE-RESPONSE LINES IN TWO DOGS
Abscissa figures when multiplied by 10^{-7} represent dose in mM per kgm Ordinate is the pressor response in mm Hg

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TABLE 1

Tests for linear regression

Description of relationship section of data used and best-fitting linear equation	Deviations: Chance n_1	Deviations: Chance Mean square	Deviations: Linear regression n_1	Deviations: Linear regression Mean square	F Observed	F For the 5 per cent point	F For the 1 per cent point	Departure from linearity
Dose vs response (Complete data) $y = 15.66 + 0.282x$	71	120 4	16	416 3	3 46	1 79	2 20	Significant
Dose vs response (Doses from 55 to 293 inclusive) $y = 13.67 + 0.343x$	38	138 2	5	102 8	0 74	2 46	3 54	Not significant
Dose vs response (U S P XI section) $y = 5.49 + 0.442x$	28	101 9	2	83 4	0 82	3 34	5 45	Not significant
Log dose vs response (Complete data) $y = -44.44 + 53.86 \log x$	71	120 5	16	2076 4	17 23	1 79	2 28	Significant
Log dose vs response (Lower dose range) $y = -2.42 + 17.55 \log x$	36	41 8	8	58 5	1 40	2 21	3 04	Not significant
Log dose vs response (Upper dose range) $y = -202.51 + 125.98 \log x$	36	201 2	6	46 5	0 23	2 38	3 38	Not significant
Log dose vs log response (Complete data) $\log y = 0.421 + 0.633 \log x$	71	0 01575	16	0 01878	1 19	1 79	2 28	Not significant

In the equations given, when values for x are multiplied by 10^{-7}, the resulting product will give the dose of epinephrine in terms of mM per kilogram, y represents the rise in systolic blood pressure in terms of mm. of Hg Under the heading "Deviation," n represents the degrees of freedom for the corresponding mean squares Under "Chance" the deviations are measured from the group mean, under linear regression the deviations are measured from the values calculated on the basis of linear regression When the term F (the ratio of the mean square of the deviations from linear regression to the mean square of the deviations within groups) is greater than the value in the 5 per cent point column, the data can be said to depart significantly from linear regression When F exceeds the 1 per cent figure the certainty of significant departure is even greater, the odds being 99 to 1 instead of 20 to 1

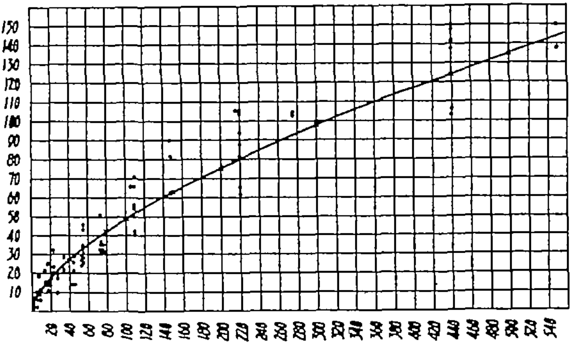

FIG 1 ARITHMETIC DOSE-RESPONSE LINE
Abscissa figures when multiplied by 10^{-7} represent dose in mM. per kgm. Ordinate is the pressor response in mm. Hg

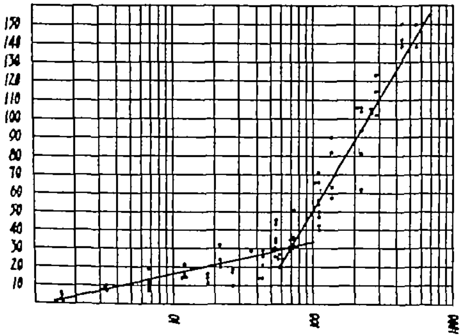

FIG 2 SEMILOGARITHMIC DOSE RESPONSE LINES
Abscissa is the logarithm of the dose. Ordinate is the pressor response in mm. Hg

selection the data should be collected from suitable animals only Since the steeper the dose-response curve the more accurate will be the results, it becomes obvious that only relatively sensitive animals should be employed

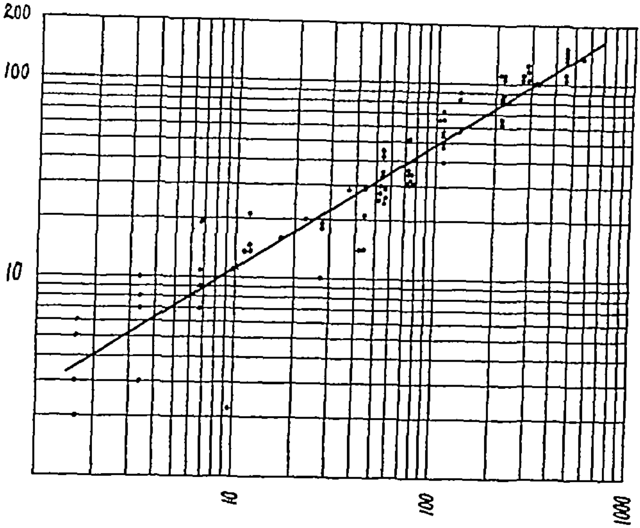

FIG 3 LOGARITHMIC DOSE-RESPONSE LINE
Abscissa is the logarithm of the dose Ordinate is the logarithm of the response

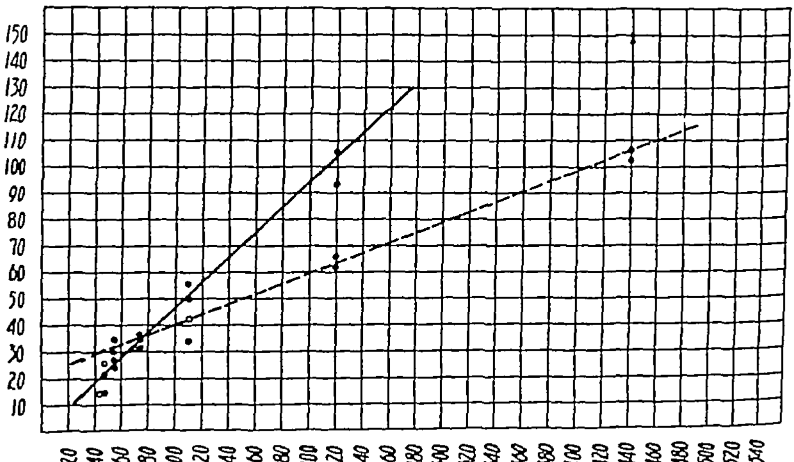

FIG 4 DIFFERENCES IN SLOPE OF ARITHMETIC DOSE-RESPONSE LINES IN TWO DOGS
Abscissa figures when multiplied by 10^{-7} represent dose in mM per kgm Ordinate is the pressor response in mm Hg

Results with other amines

Ten and eight dogs were used respectively, in the investigation of the pressor effects of *3 4*-dihydroxyphenethylmethylamine and *3 4*-dihydroxyphenethyldimethylamine a member of a series of tertiary phenethylamines prepared by Buck *et al* (8) When the data collected with each of these two compounds were treated statistically in the same manner as were the epinephrine data, similar curves were obtained which differed only in the steepness of the respective logarithmic curves and in their position with respect to the two coordinate axes The relative results are summarized in table 2. These results serve to emphasize the statement made before that any compound which is to be compared with epinephrine for its relative pressor potency should be tested in accordance with a method of the character of the

TABLE 2

Relative potencies of the three amines at three different response levels

COMPOUND	SLOPE OF LOG CURVE	DOSE* AT VARIOUS RESPONSE LEVELS					
		30 mm. Hg	r	65 mm. Hg	r	100 mm. Hg	
Epinephrine	0 633	46		158		312	
3 4-Dihydroxyphenethylmethylamine	0 740	530	11 5	1500	9.5	2600	8 3
3 4-Dihydroxyphenethyldimethyl amine	0 795	630	13 7	1650	10 4	2900	9 3

Multiplying dose figures by 10^{-7} gives mM per kilogram r signifies ratio between the particular amine and epinephrine derived by dividing dose of the amine by the dose of epinephrine at each response level.

one herein employed. If the slopes of the dose-response curves are not identical, the relative potencies of any two compounds will differ according to the response value at which comparison is made (see table 2) Under this condition the relative potencies can only be expressed in terms of the doses required to elicit a certain response supplemented by the determined values of the slopes.

DISCUSSION

Lyon (4) interpreted his results as obeying the Weber Fechner law Wilkie (5) corroborated Lyon s observations in the higher dose range, but claimed that with lower doses there is a linear relationship between dose and response Rosenblueth (6), following the Elliott (2) procedure, stated that the dose-response curve was a rectangular hyperbola. Lyon s data in Rosenblueth s hands gave not a linear dose-response relationship but an S-curve This difference of opinion is readily understandable for, as Clark (9) has pointed out, only minor variations in data often enable the fitting of several different curves Our own data indicate that linear dose-response relationships can

be shown to exist depending upon the sections of data selected and upon their mathematical expression and that the Weber-Fechner law holds for certain restricted data but not for the whole range of data When a section of data is selected between dose values of 55 $\times$ 10^{-7} to 293 $\times$ 10^{-7} mM per kilogram, a linear dose-response relationship is shown on arithmetic treatment This response range includes that specified by the U.S P.XI In view of the fact, however, that we have encountered varying sensitivities in different animals to progressively increasing doses it is probable that the U S P XI procedure could be improved by either using sensitive dogs only or perhaps by even sensitizing test animals prior to experiment with some such drug as cocaine

The linear logarithmic dose-response relationship demonstrated for epinephrine, *3,4*-dihydroxyphenethylmethylamine and *3,4*-dihydroxyphenethyldimethylamine suggests that its determination might prove generally useful for the comparison of the relative potencies of sympathicotonic substances Since within certain restricted limits the arithmetic dose-response curve is also a straight line, an alternative to the foregoing would be the comparison of the unknown with epinephrine, when possible, in the same, preferably sensitive, dog by alternate progressively increasing doses,

SUMMARY

Three pressor amines, namely, epinephrine, *3,4*-dihydroxyphenethylmethylamine and *3,4*-dihydroxyphenethyldimethylamine were tested for relative potencies by an increasing dose method, and the data were studied to determine when linear relationships might be assumed The following conclusions may be drawn

1 The Weber-Fechner law may be said to hold for restricted ranges of our data but not for the data as a whole

2 The application of a progressively increasing dose method to the biological assay of epinephrine may be an improvement upon the U S P XI procedure

3 Expressing relative pressor potencies on the basis of doses required to elicit a specific response may not convey a true picture when the characteristic slopes of the logarithmic dose-response curves of two compounds are not identical

4 When the logarithmic curves of compounds being compared with epinephrine are dissimilar, it may indicate the presence of qualitative differences in action

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STUDIES ON PURIFIED DIGITALIS GLUCOSIDES[1]

III THE RELATIONSHIP BETWEEN THERAPEUTIC AND TOXIC POTENCY

McKEEN CATTELL AND HARRY GOLD

From the Department of Pharmacology, Cornell University Medical College, New York

Received for publication August 10, 1940

A point of practical as well as theoretical interest lies in the answer to the question of whether the digitalis glucosides possess differences in toxicity in relation to their therapeutic potency There are numerous references in the clinical literature, especially European reports, which testify to the widely held view that one or another of the digitalis preparations is superior in the treatment of this or that cardiac disorder, and it is not infrequently suggested that toxic manifestations call for the substitution of some other digitalis glucoside

There is nothing in our knowledge of the pharmacology of the digitalis glucosides to justify the *a priori* assumption that one preparation possesses special superiority over another with regard to the spread between the therapeutic dose and the dose causing toxic symptoms Nor is this thesis supported by any well controlled clinical or laboratory comparisons Moe and Visscher (1) have recently reported the results of experiments in which several glucosides from *Digitalis Lanata* (lanatoside A, B and C) were compared in the heart-lung preparation of the dog, and from which they deduced that the ratio of toxic to therapeutic dose was much the highest for lanatoside C These findings will be discussed later, but it may be mentioned now that we have not been able to find in the literature convincing evidence of differences in the ratio of toxic to therapeutic dose among the digitalis principles Much of the existing evidence is derived from toxic doses obtained in animal experiments in relation to the therapeutic dose in man, a comparison which is not justified in view of the differences in relative potency obtained when different test objects are employed (Cattell and Gold, 2)

In the present report we present comparative data on the toxic effects in relation to therapeutic potency of several of the digitalis glucosides using a single isolated tissue as the test object—the papillary muscle from the right side of the cat heart

[1] We are indebted to Mr Charles Salzman for assistance in the performance of the experiments, and to the Laboratoire Nativelle, Paris, Merck & Co, Schieffelin & Co, Sandoz Chemical Works and the Warner Institute for Therapeutic Research for materials and other aid in connection with these studies

METHOD

The general procedures employed were the same as those described in an earlier study (Cattell and Gold (3)) The isolated papillary muscle from the right side of the cat heart was connected to an optically recording isometric lever The muscle was maintained in 20 cc. of a modified Locke s solution (NaCl 0 9 per cent KCl 0 042 per cent, $CaCl_2$ 0 012 per cent, glucose 0 1 per cent, phosphate 0 01 per cent to give pH 7 2) at 37°C through which oxygen was slowly passed and to which the digitalis glucosides were added to give the desired concentration. In order to avoid the possibility of complication due to the persistence of action of these substances only a single application was made to any one preparation In certain experiments blood serum was substituted for the Locke s solution. By means of a thyratron stimulator condenser

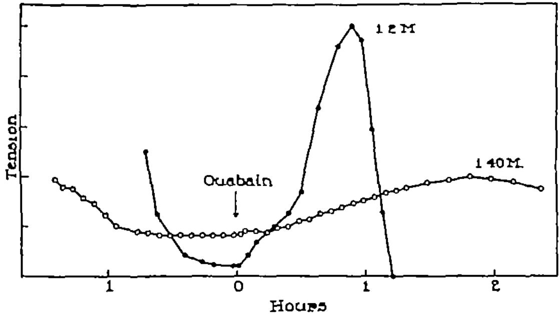

FIG 1. TYPICAL EXPERIMENTS SHOWING THE EFFECT OF OUABAIN ON THE CONTRACTION OF PAPILLARY MUSCLES IN THERAPEUTIC CONCENTRATION (OPEN CIRCLES) AND TOXIC CONCENTRATION (SHADED CIRCLES)

discharges were applied to the muscle at a constant rate (40 to 50 per minute) throughout the duration of the experiment. The activity of the muscle (isometric contractions) was recorded at frequent intervals (usually every 5 or 10 minutes) throughout the course of the experiment, which lasted several hours. From these records a plot was made of the course of the tension response including the period before adding the digitalis glucoside and for about 5 hours following, unless the contraction had ceased in the meantime

In order to gain evidence on the question of whether there is a difference in the ratio of toxic to therapeutic dose among the various digitalis preparations a first requisite was the selection of criteria for these two effects. A prominent influence of digitalis is that of increasing the force of contraction of the isolated papillary muscle (Cattell and Gold (3)) a change which undoubtedly represents the therapeutic action in man The concentration of digitalis glucoside which was effective in producing an augmentation in the response in approximately 50 per cent of the experiments was arbitrarily chosen in the present study as representative of the minimal therapeutic dose or as we shall refer to it the *therapeutic factor* When the muscle is exposed to considerably

higher concentrations of the digitalis glucosides, toxic effects soon occur After an initial augmentation the contractile tension begins to fall off and soon fails altogether During this period other toxic effects are present, such as extra (spontaneous) contractions, and missed responses (raised threshold for excitation) The *toxic factor* which we used in the final calculations was determined by the time it takes from the application of the drug through the period of augmentation to the subsequent fall to one half the maximum tension

These criteria will be made clear by reference to figure 1 which shows typical effects of therapeutic and toxic concentrations of ouabain The light circles illustrate the course of an experiment in which a moderate dose of the drug was employed The force of contraction increases gradually under the influence of ouabain and is well maintained, usually for at least 5 to 8 hours The curve designated with solid circles is typical of the effects on contraction of a toxic concentration of the digitalis glucosides—in this instance 1 part of ouabain in 2 millions of solution Such a graph was plotted for each experiment and from them measurements were made, constituting the data presented in the tables

The procedure was as follows First the threshold concentration resulting in augmentation was established in a series of preparations (therapeutic factor) Other preparations were exposed to toxic concentrations of the same drug producing effects within a convenient time limit (toxic factor)—usually between 1 and 3 hours The toxic concentrations which were used for the comparison of various glucosides were multiples of the therapeutic concentrations, usually between 20 and 50 times the therapeutic concentrations In the present communication data are given for ouabain (Merck), digitoxin (Merck) and "Digitaline Nativelle" (Laboratoire Nativelle) and lanatoside A, B and C (Sandoz)

RESULTS

The data from which the estimation of minimal therapeutic concentrations (therapeutic factor) was derived for ouabain, digitoxin and lanatoside A, B and C, based on more than 100 experiments, are given in figure 2 Approximately half of the preparations gave a positive response (increased force of contraction) at dilutions of ouabain and digitoxin of 1 part in 100 millions[2] Thus these two glucosides possess a similar order of therapeutic potency

The results for the lanatoside compounds (fig 2) show them to be much less potent, i e they have only about a tenth of the activity of ouabain or digitoxin in the isolated papillary muscle

The results for the several lanatoside compounds are designated by different symbols in the figure, from which it will be seen that lanatoside A, B and C do not differ appreciably with regard to their activity in augmenting the concentration of the papillary muscle preparation, i e , their therapeutic potency as here defined is similar

Data relating to the toxicity of the several glucosides investigated are assembled in tables 1, 2 and 3 Figures are given for the time from the application of the drug in various concentrations to *1* the maximum augmentation

[2] Occasional positive responses have been observed at dilutions as high as 1 part in 150 and 200 millions

of the tension response, *2* the subsequent reduction to 50 per cent of the maximum response and *3* the complete loss of contractility The tables also include data in the third column, showing the degree of augmentation occurring in each experiment, expressed as a multiple of the tension present at the time the glucoside was administered. Since this figure is influenced by the original magnitude of the response and other factors, it is not a reliable measure of potency of the drugs A number of determinations have been

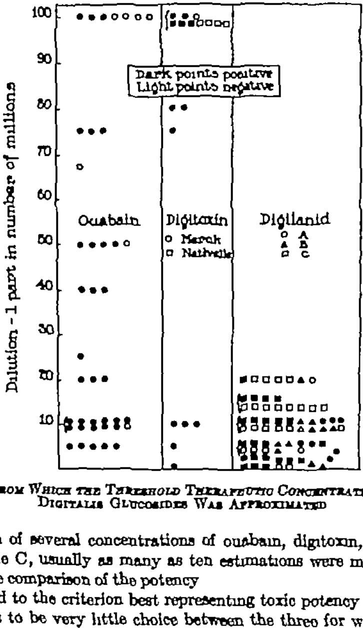

FIG 2. DATA FROM WHICH THE THRESHOLD THERAPEUTIC CONCENTRATION OF SEVERAL DIGITALIS GLUCOSIDES WAS APPROXIMATED

made at each of several concentrations of ouabain, digitoxin, lanatoside B and lanatoside C, usually as many as ten estimations were made at points critical for the comparison of the potency

With regard to the criterion best representing toxic potency (toxic factor) there appears to be very little choice between the three for which data are given in the tables. The time elapsing before the maximum response is reached might be influenced by the rate at which the therapeutic effect is produced and therefore is not determined solely by the toxic action responsible for the following decline The time to complete failure is likely to be some-

TABLE 1

Time to onset of effects produced by toxic concentrations of ouabain

EXPERIMENT DATE	CONCENTRATION	AUGMENTATION*	MAX.†	½ MAX.‡	END§
		multiple	*minutes*	*minutes*	*minutes*
2/23/40	1:1,000,000	32	25	32	40
4/ 9/40		40	19	26	29
4/10/40		15	34	45	56
4/11/40		6	25	37	50
4/11/40		6	34	51	69
Average			27.4	38.2	48.8
Standard deviation			2.1	4.4	2.1
2/23/40	1:2,000,000	6	47	80	112
2/27/40		8	54	74	84
4/ 1/40		9	60	77	92
4/ 2/40		3½	86	117	136
4/ 4/40		36	83	100	113
6/ 5/40		5	66	93	109
6/ 6/40		2	49	70	84
6/ 6/40		3	69	102	124
6/ 6/40		4	40	69	80
6/ 8/40		6	99	113	129
Average			65.3	89.5	106.3
Standard deviation			6.0	5.6	6.3
2/26/40	1:5,000,000	2½	124	194	230
4/ 3/40		1¼	121	138	154
4/ 3/40		2	174	204	249
4/ 4/40		16+	120	218	266
4/ 5/40		2	106	146	191
5/ 7/40		5	85	108	130
5/ 9/40		4	67	124	147
5/15/40		2	29	70	76
5/21/40		3	79	94	124
5/23/40		10	45	80	125
5/24/40		3½	150	186	229
5/27/40		8	109	136	149
5/27/40		7	70	97	120
Average			98.4	138.1	168.5
Standard deviation			11.4	13.8	16.3

* Numbers represent maximum tension in multiples of the tension preceding the application of the drug.

† Interval between application of drug and rise to maximum tension.

‡ Interval between application of drug and fall to ½ maximum tension.

§ Interval between application of drug to loss of contractility.

what drawn out and thus not representative of a sharp end-point. The figures for the time required for the tension to fall to one-half the maximum, on the other hand represent a point of rapid change and theoretically should give the most accurate measurement As already stated, this is the one (toxic factor)

TABLE 2

Time to onset of effects produced by toxic concentrations of Digitaline Nativelle'

EXPERIMENT DATE	CONCENTRATION	AUGMENTATION	MAX.†	½ MAX.‡	END§
		multiple	*minutes*	*minutes*	*minutes*
3/ 6/40	1 2 000 000	12	77	81	92
3/ 6/40		22	66	118	183
3/27/40		3½	51	79	91
3/28/40		34	88	98	128
3/28/40		10	40	70	86
6/ 4/40		5	71	99	121
6/ 4/40		10	57	70	86
6/ 4/40		9	76	86	113
6/ 4/40		3	58	65	83
6/ 5/40		16	76	94	108
Average			66 0	86 0	104 1
Standard deviation			4 5	5 3	5 6
3/25/40	1 5 000 000	9	99	133	169
3/25/40		5	90	146	158
3/26/40		40+	67	93	112
3/26/40		7	100	117	150
3/26/40		14	116	150	184
5/16/40		2	61	72	76
5/16/40		6	104	155	174
5/18/40		9	88	107	156
5/22/40		1½	93	124	146
5/22/40		2	141	174	186
Average			95 9	127 1	151 1
Standard deviation			7 1	9 1	11 6

Numbers represent maximum tension in multiples of the tension preceding the application of the drug.

† Interval between application of drug and rise to maximum tension.

‡ Interval between application of drug and fall to ½ maximum tension.

§ Interval between application of drug to loss of contractility

on which the final ratios were based. Actually, however these three points fall on an approximately straight line differing only in slope when the average times are plotted This is true for every group of experiments so that any one of these points gives the same answer with reference to the toxicity of the drug in question

The validity of the toxic factor which we used, namely the interval from

TABLE 3

Time to onset of effects produced by toxic concentrations of lanatoside C and B

EXPERIMENT DATE	CONCENTRATION	AUGMENTATION*	MAX.†	½ MAX.‡	END§
		multiple	*minutes*	*minutes*	*minutes*
4/ 4/40	1 200,000 Lanatoside C	15	29	45	57
2/29/40		36	18	25	29
2/29/40		4	25	33	38
2/29/40		10	39	53	56
4/ 5/40		13	46	55	76
Average			30 6	40 2	51 2
Standard deviation			5 0	5 8	8 1
3/ 1/40	1 500,000 Lanatoside C	14	58	67	81
3/ 1/40		33	66	87	110
3/ 6/40		15	66	87	96
3/ 6/40		6½	46	64	74
3/ 7/40		7½	48	78	96
3/22/40		6+	59	74	79
3/22/40		8	50	89	119
3/22/40		8	46	60	64
3/23/40		11	50	61	95
3/25/40		5	77	107	120
Average			56 6	77 4	93 4
Standard deviation			1 1	5 6	6 0
3/ 5/40	1 500,000 Lanatoside B	11	27	40	52
3/ 5/40		4	44	60	75
3/ 5/40		2½	36	64	94
3/ 7/40		4	33	44	53
3/ 7/40		3	30	60	80
3/ 7/40		9	61	103	110
3/19/40		4	59	68	94
3/19/40		8	36	78	97
3/21/40		3	36	54	71
3/21/40		5	35	55	90
Average			39 7	62 6	81 6
Standard deviation			3 7	5 7	6 3
4/ 8/40	1 750,000 Lanatoside C	2+	49	59	109
4/ 8/40		3	84	149	184
4/ 8/40		7	59	74	92
4/ 8/40		6	69	92	99
4/ 9/40		2½	74	119	134
Average			67 0	98 6	123 6
Standard deviation			6 0	16 1	17 3

* Numbers represent maximum tension in multiples of the tension preceding the application of the drug

† Interval between application of drug and rise to maximum tension

‡ Interval between application of drug and fall to ½ maximum tension

§ Interval between application of drug to loss of contractility

the time of application of the drug to the time when the systolic tension was reduced to 50 per cent of the maximum, as an expression of potency, was established by the comparison of different concentrations of each glucoside, the data for which are presented in the tables It will be seen that the time is prolonged in direct relation to the reduction in concentration and that this criterion serves as a fairly sensitive biological indicator of the potency of the drug in the range employed An analysis of the data indicates that a change in potency of 50 per cent can be recognized without difficulty Even though fairly large individual variations occur within each group, when the number of experiments is increased to ten the standard deviation from the average is less than 10 per cent

The first point of interest lies in the comparison of the ratio of the toxic to therapeutic dose in the several members of the lanatoside series, since it has been suggested by Moe and Visscher (1) that in this regard lanatoside C may have as much as a 30-fold superiority over lanatoside B The data of figure 2 already referred to, fail to indicate differences in potency among the three lanatoside compounds A, B and C with regard to their action in improving the force of contraction of cardiac muscle, i.e. their therapeutic potencies, as here defined, are similar A comparison of the toxic potency of lanatoside C and lanatoside B is given by the data in table 3 A slight superiority is suggested for the former in concentrations of 1 part in 500 000 since it took about 25 per cent longer to reach the point where the tension fell to 50 per cent of the maximum. This difference is mathematically not significant That in any case it would represent something less than a 50 per cent greater toxic potency for lanatoside B over C was shown by additional experiments with lanatoside B employing a 50 per cent higher dilution—a dilution which, on the basis of the results given in table 3, was definitely less toxic than 1 500,000 of C Thus the ratio of toxic to therapeutic concentration of lanatoside C and lanatoside B do not differ materially and fail to support the conclusion reached by Moe and Visscher (1) derived from experiments on other material

It was not possible to distinguish between ouabain and digitoxin on the basis of their action on the papillary muscle For both, the threshold concentration, i.e., that augmenting the response in 50 per cent of the experiments, was approximately 1 part in 100 millions of Locke s solution (see fig. 2) The toxic effects of stronger solutions (tables 1 and 2) appeared at the same time with similar concentrations of the two drugs within the accuracy of the measurements. Thus the ratio of toxic to therapeutic concentration was found to be the same for ouabain and digitoxin.

The most important difference appears when a comparison is made of the lanatoside group with ouabain and digitoxin. The minimal therapeutic concentration may be taken as 1 to 10 million for the lanatosides and 1 to 100 million for the others (fig. 2) With a 50-fold increase in the therapeutic

dose (1 2,000,000) the toxic factor which we used appeared in 89 minutes for ouabain and 86 minutes for "Digitaline Nativelle" (tables 2 and 3), while with a corresponding 50-fold increase in the therapeutic dose of lanatoside C (1 200,000) the toxic factor was reached in an average time of only 40 minutes Also, with a 20-fold increase in the therapeutic dose (1 5,000,000) the toxic factor appeared in 138 minutes for ouabain and in 127 minutes for "Digitaline Nativelle," whereas, with a corresponding 20-fold increase in the therapeutic dose of lanatoside B and C (1 500,000) the toxic factor was reached in 63 and 77 minutes Thus the spread between the toxic and therapeutic doses is somewhat smaller for the lanatosides than for "Digitaline Nativelle" or ouabain viz ouabain and digitoxin have a toxic to therapeutic ratio of between 2 and 3 times that of lanatoside B and lanatoside C This difference is not large, and until confirmatory results are obtained a final conclusion is not justified

DISCUSSION

The problem of central interest in the present investigation rests in the answer to the question—Do the various digitalis glucosides differ among themselves with respect to the ratio of toxic to therapeutic dose? In a simple tissue such as cardiac muscle, in the absence of evidence to the contrary, it would appear likely that the property of the glucoside responsible for the therapeutic action would also mediate the toxic effects which appear when the concentration is sufficiently raised If all the cardiac glucosides act through a similar mechanism it should then be impossible to dissociate these effects by changes in the structure of the molecule, i e , the spread between therapeutic and toxic doses for all should be the same The situation is more complex in the intact animal where theoretically side effects, such as emesis, might more probably be mediated by a portion of the molecule not essential to its therapeutic action

Conclusive experimental evidence bearing on this problem is wanting Moe and Visscher (1) have investigated the question, utilizing efficiency changes in the heart-lung preparation of the dog as an index of therapeutic action, and have arrived at the conclusion that there are wide differences in the ratio of therapeutic to toxic doses among digitalis glucosides By relating the fatal intravenous dose in the cat to the dose causing efficiency changes in the heart-lung preparation of the dog they deduced that the ratio of toxic to therapeutic dose is about 30 times as large for lanatoside C as for lanatoside B However, in view of the great divergence in susceptibility obtaining from one animal to another, and in the same animal depending on the experimental set-up (see Cattell and Gold (2)) this comparison is not justified Examples illustrating the danger of drawing conclusions from such comparisons are to be found in the data of this paper Thus ouabain and digitoxin have the same potency when tested on the papillary muscle, but when assayed

by the Hatcher Brody cat technic the potency of ouabain is about 4 times that of digitoxin, and by the one-hour frog method it is about 8 times that of digitoxin The lanatoside compounds have only about a tenth of the activity of ouabain or digitoxin on the papillary muscle of the cat, but in the intact animal lanatoside C is more potent than digitoxin.

Moe and Visscher (1) also related the dose required to produce cardiac irregularities to the efficiency improving dose in the heart lung preparation and obtained results leading to the curious conclusion that lanatoside A and B, but not C, produce toxic effects in doses smaller than those required to produce therapeutic effects When by this method, the ratio of the toxic to therapeutic dose was computed for the several members of this group, lanatoside C gave a figure at least 9 times that of lanatoside B and almost as great for lanatoside A Since both the toxic and therapeutic doses were measured on the same biological material this is a more significant type of comparison However, further study is needed to establish the value of the ratios, for while the doses were arrived at by taking the smallest amount producing a positive effect in a series of experiments fairly large increments of dosage were used and the values for the minimal effective therapeutic and toxic doses were determined in the case of lanatoside C by single observations

Ten years ago a study was made by Gold Hitzig Gelfand and Glassman (4) in an endeavor to answer the same question and this led to a conclusion opposite to that of the more recent work of Moe and Visscher Comparisons were made for a number of digitalis bodies with reference to the average percentage of the fatal dose producing the first change in various electrocardiographic signs in cats They stated " we have not examined all the actions of the digitalis bodies not even the most important one, namely, the action directly upon the heart which improves myocardial efficiency in congestive heart failure We have, however, compared a number of cardiac actions within both the therapeutic and toxic range of widely different digitalis bodies and the results show that these act with the same relative intensity upon different cardiac structures. This conclusion gains strong support from some recent studies on man (Gold Kwit and Cattell (5) Kwit Gold and Cattell (6)) which indicate that changes in the T wave of the electrocardiogram run parallel with the essential therapeutic action of digitalis which leads to changes in the heart rate of patients with auricular fibrillation These papers also present evidence on the relationship between the toxic and therapeutic dose in man for digitalis, Digitaline Nativelle and lanatoside C (Cedilanid Sandoz) The data are summarized in table 4 These results indicate that the therapeutic dose for each of the three preparations represents approximately the same percentage of the toxic dose

The experimental evidence discussed above leaves considerable doubt as to whether the toxic effects of the digitalis glucosides can be dissociated from their therapeutic effects, it is not unlikely that they are both manifestations of

the same fundamental action on the cell For the most part the results of the present study support this thesis On the papillary muscle of the cat ouabain and digitoxin have about the same potency with reference to both their therapeutic and toxic effects Similarly the lanatoside glucosides A, B and C, while possessing a lower potency, do not differ among themselves with regard to the spread between therapeutic and toxic concentrations However, there is an interesting difference between these two groups According to the criteria used ouabain and digitoxin appear to be slightly less toxic in relation to their therapeutic concentrations than are the lanatoside compounds, viz in the average the ratio of the toxic to therapeutic dose is between 2 and 3 times greater for ouabain and digitoxin In view of its theoretical and practical implications we wish at the present time to leave open the question of the significance of this small difference We expect to obtain further data with a modified technic which it is hoped will give a more precise answer

TABLE 4

Ratio of toxic to therapeutic dose in man

	PERCENTAGE OF FULL THERAPEUTIC DOSE	NUMBER OF CASES	INCIDENCE OF TOXICITY
			per cent
Digitalis	100	82	3 7
Digitalis	200	125	38 4
Digitaline (Nativelle)	100	24	8 3
Digitaline (Nativelle)	200	13	46 0
Lanatoside C	100	26	7 6
Lanatoside C	200	16	50 0

SUMMARY

The relative potency with regard to both therapeutic and toxic effects on isolated papillary muscles from the cat have been determined for the following glucosides Ouabain (Merck), Digitoxin (Merck), Digitaline (Laboratoire Nativelle), Lanatoside A (Sandoz), Lanatoside B (Sandoz) and Lanatoside C (Cedilanid, Sandoz)

The minimum concentration of ouabain and digitoxin, i e, that causing increased force of contraction in 50 per cent of experiments (therapeutic effect) is 1 part in 100 millions of Locke's solution, for the lanatoside compounds it is approximately 1 part in 10 millions

When the concentration of any of the glucosides is sufficiently increased toxic effects are produced, including extra contractions, loss of excitability and diminished force of contraction

The ratio of the concentrations producing therapeutic and toxic effects is the same for "Digitaline Nativelle" and ouabain There is also similarity among the several lanatoside glucosides studied and thus by this technic we have not been able to confirm the wide margin between the therapeutic and toxic dose reported by Moe and Visscher for lanatoside C.

Ouabain and "Digitaline Nativelle" both give a slightly higher value for the ratio of toxic to therapeutic concentration in comparison with the lanatoside compounds. The evaluation of the significance of this finding must await further evidence.

The available evidence pertaining to the questions of differences in the relationship between toxic and therapeutic doses among different glucosides is discussed, and the conclusion is reached that proof of the existence of such differences is still wanting

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ANESTHESIA[1]

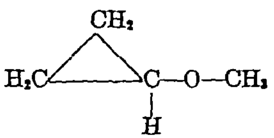
III THE PHARMACOLOGY OF METHYL ALLYL ETHER

JOHN C KRANTZ, JR , C JELLEFF CARR, SYLVAN FORMAN AND W G HARNE

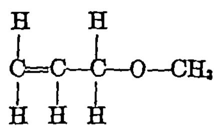
From the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore

Received for publication October 23, 1940

Previous studies in this laboratory have shown the value of cyclopropyl methyl ether (cyprome ether) as an anesthetic agent in several species of animals (1) Furthermore, anesthesias have been conducted on man and various operative procedures have been carried out satisfactorily under this anesthetic agent (2) Methyl allyl ether, which is a structural isomer of cyprome ether, differs from the latter only by the presence of a double bond instead of a three membered ring in the molecule The chemical relationship is shown by the formulae



Cyprome Ether



Methyl Allyl Ether

Owing to this constitutional relationship, it seemed desirable to study the pharmacology of methyl allyl ether All experimental technics employed in this investigation were the same as those used with cyprome ether

OBSERVATION ANESTHESIAS

General behavior

Ten dogs were anesthetized with methyl allyl ether The induction period of anesthesia was long and the animals struggled considerably before passing into the plane of surgical anesthesia Salivation was quite marked during induction Surgical anesthesia was uneventful, but complete relaxation of the musculature of the abdomen or the extremities was not obtained There

[1] The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Co of Cleveland, Ohio The authors are greatly indebted to Dr Amos G Horney of the technical staff of this company for preparing large quantities of methyl allyl ether for the prosecution of these studies

were many incoordinated movements of the legs during anesthesia. Pain reflexes were abolished. The period of recovery from anesthesias of 20 to 30 minutes duration was short, but longer than that observed with ether. During recovery the animals showed violent incoordinated leg and head movements. Tremors and extensor rigidity of the hind legs were observed. The head was frequently thrust forcefully against the floor of the cage in a severe syndrome of sneezing. The sneezing in some animals occurred periodically for two days after the anesthesia. In others it developed some

TABLE 1

Anesthetic index in dogs—allyl methyl ether

NUMBER	WEIGHT OF DOG	SEX	ANESTHETIC INDEX	INDUCTION	RESPIRATORY FAILURE
	kgm.			*cc. per kgm.*	*cc. per kgm.*
1	7.0	M	1.16	1.79	2.07
2	9.5	M	1.50	1.05	1.58
3	11.9	M	2.00	1.39	2.73
4	6.1	F	1.80	0.90	1.64
5	6.2	M	1.26	1.85	2.30
6	7.9	M	1.21	1.46	1.77
7	6.9	M	1.75	0.87	1.52
8	11.0	M	2.30	1.18	2.70
9	7.5	F	1.41	1.12	1.60
10	7.9	M	1.93	1.01	1.96
11	7.1	M	1.93	1.13	2.18
12	7.4	F	1.55	1.21	1.00
13	12.2	F	1.62	0.86	1.40
14	9.4	F	1.43	1.22	1.75
15	10.8	M	1.96	1.16	2.27
16	9.5	M	1.48	1.21	1.79
17	5.3	F	1.46	1.04	1.51
18	10.0	M	1.70	0.85	1.45
			1.63	1.18	1.89
σ			0.30		

time after recovery and lasted for 24 to 48 hours. The buccal and nasal membranes in dogs appeared inflamed for several days.

Anesthetic index

The number of cubic centimeters of the anesthetic required to produce surgical anesthesia was divided into the volume required to produce respiratory arrest and the quotient designated as the anesthetic index. The results are set forth in table 1.

The anesthetic index is slightly less than that observed with ethyl ether,

the index of which is 1 76 Sigma, however, was much larger in this series of experiments, owing to the difficulty experienced in differentiating the anesthetic planes The volumes required for induction and respiratory failure were not significantly different from those employed in ethyl ether experiments With cyprome ether, however, approximately half of this volume was required for induction

Blood pressure

The effect of methyl allyl ether on the blood pressure of the dog was determined by three typical experiments Blood pressure fell progressively from the beginning of surgical anesthesia The rate and amplitude of respiration also were depressed and respiration failed simultaneously with cardiac stoppage

TABLE 2

Induction concentration in mice—methyl allyl ether

PARTIAL PRESSURE	CC. PER LITER	NUMBER OF MICE PER JAR	NUMBER OF MICE USED	PER CENT ANESTHESIA	AVERAGE INDUCTION TIME
per cent					*minutes*
3	0 12	3	12	0	
4	0 16	3	12	25	7
5	0 20	3	12	67	7
6	0 24	3	36	92	5

Effect on the perfused heart of the frog

Methyl allyl ether was dissolved in Howell-Ringer's solution and perfused through the frog's heart in situ Previously it had been shown that the isomer, cyprome ether, could be perfused through the frog's heart in 0 014 M concentrations for at least 30 minutes without demonstrable effect This concentration of methyl allyl ether caused immediate diminution of the amplitude of the beat A 0 1 M solution of methyl allyl ether produced cardiac stoppage within one minute Methyl allyl ether therefore exhibits a greater cardiac toxicity than does cyprome ether Twelve experiments were conducted

Concentration required for anesthesia in mice

The concentration in the respired air required to induce anesthesia was determined by typical partial pressure experiments on mice The results are set forth in table 2

The animals showed marked lacrimation and irritation of the upper respiratory tract during anesthesia Within 5 days after anesthesia, half the animals died The threshold concentrations for anesthesia in the mouse appear to be approximately the same for methyl allyl ether and cyprome ether How-

ever, the induction period is approximately one-tenth as long for the latter There were no deaths attributable to the anesthetic when cyprome ether was employed.

Histological studies of liver and kidney tissue in rats

Six white male rats weighing between 175 and 200 grams were anesthetized for 10 minutes on 5 successive days During the final anesthesia the liver, kidneys and lungs were extirpated and then examined histopathologically by Professor Hugh R. Spencer of the Department of Pathology

The lungs and kidneys of all of the animals appeared to be normal. The livers showed necrotic changes of the central portion of the lobules involving from one-third to one-half of the tissue of the lobule

DISCUSSION

Although the pharmacologic action of methyl allyl ether has not been previously investigated the action of many different compounds containing the allyl group have been studied (3) Allyl alcohol is strikingly irritant to the mucous membranes and will produce convulsions, coma and death. The alcohol is reputed to be fifty times more toxic than propyl alcohol its saturated analogue (4) The irritating properties of the allyl group upon the mucous membranes apparently are exhibited by methyl allyl ether We believe that the allyl group is responsible for the protracted sneezing and nasal irritation accompanying anesthesia with this compound. A sample of methyl allyl ether was washed well with water, dried and treated with sodium hydroxide to remove any possible traces of allyl alcohol. This sample still produced the characteristic post-anesthetic syndrome. In addition a dog was anesthetized with ethyl ether containing 0.5 per cent allyl alcohol and the syndrome following anesthesia with methyl allyl ether did not occur These experiments show that if a small amount of allyl alcohol were present in methyl allyl ether it could not have been the causative factor of the post anesthetic syndrome.

SUMMARY AND CONCLUSIONS

1 Methyl allyl ether, isomeric with cyclopropyl methyl ether, exhibits anesthetic properties in the dog rat and mouse.

2 This pharmacologic study of methyl allyl ether, in our opinion shows that its toxicity to the liver and its devastating post-anesthetic syndrome render it unsuitable as an anesthetic in man.

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STUDIES OF THE URINARY EXCRETION OF GUANIDINE ADMINISTERED ORALLY TO NORMAL PERSONS AND PATIENTS WITH MYASTHENIA GRAVIS[1]

A S MINOT AND HELEN E FRANK

From the Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee

Received for publication November 8, 1940

In our report (1) that guanidine hydrochloride causes marked symptomatic improvement in myasthenia gravis we noted that persons with this disease tolerate considerably larger doses of the drug than do normal individuals The greater tolerance appeared to be due to a greater ability to take guanidine without producing a sustained increase in the amount present in the blood rather than to a decreased sensitivity to the effect of increased concentration of guanidine in the body fluids When sufficiently large doses of the drug are given to cause a persistent significant elevation of the amount of guanidine in the blood of persons with myasthenia gravis they manifest the same symptoms of intolerance as are shown by normals with a comparable degree of hyperguanidinemia The striking difference is the comparative doses required to cause equal increases in the level of guanidine in the blood of normal individuals and of persons with myasthenia gravis

This observation raises certain simple preliminary questions which are of fundamental importance in an analysis of the mechanism of the effectiveness of guanidine as a therapeutic agent Do persons with myasthenia gravis absorb the drug as promptly and completely as normal persons? Do they rapidly excrete unchanged a larger proportion of the amount absorbed? Do their tissues pick up from the blood stream and retain larger amounts of guanidine? Do they more rapidly convert guanidine into other compounds which are non-toxic?

Our earlier demonstration in patients with myasthenia gravis that the promptness of response and the effectiveness of a given amount of guanidine is nearly the same whether the drug is given orally or intravenously indicates a rapid and nearly complete absorption of guanidine hydrochloride from the gastrointestinal tract

The main purpose in the present investigation was to compare the urinary excretion of ingested guanidine in patients with myasthenia gravis and in normal persons In the hope of throwing some light on the problem of the

[1] This work was aided by a Grant from the John and Mary R Markle Foundation

possible conversion of guanidine into other related compounds we also studied the effect of guanidine administration on the urinary excretion of creatine and glycocyamine (guanido acetic acid) Thompson (2) has reported a marked increase in creatine excretion following the simultaneous administration of guanidine and hexamethylene-tetramine. Wishart (3) found an increase in muscle creatine in animals following the administration of guanidine carbonate and Beard and Pizzolato (4) made similar observations following the injection of methyl guanidine in rats. Evidence is constantly accumulating (5–8) that glycocyamine is a probable intermediate product in the synthesis of creatine in the body

Considerable preliminary work was necessary in order to work out satisfactory procedures for the quantitative determination of guanidine and glycocyamine in urine. A brief description of the methods used together with the experimental data obtained are presented in this paper

METHODS

The determination of guanidine involves separation from the chemically related compounds creatine and creatinine which are present in considerable amounts The procedure which we finally adopted was a combination of the separation of guanidine as picrate according to the method of Sharpe (9) followed by a colorimetric determination of the precipitated guanidine by the method of Sullivan (10) In brief Sharpe precipitates from an aqueous solution and weighs as picrate the guanidine which has been removed by alcoholic extraction from the dried residue obtained by the evaporation of a considerable volume of urine previously treated with tannic acid and barium hydroxide. The first picrate precipitate obtained is purified by recrystallization in order to remove creatinine and ammonium picrates which are present as contaminants The method has been subjected to criticism by Greenwald (11) and White (12) on the basis that picrates other than guanidine may persist after recrystallization and add to the apparent guanidine determined gravimetrically Rather than weighing the picrate obtained we have found it more satisfactory for our purpose to take advantage of Sullivan s colorimetric method for the determination of the guanidine present in a solution of the picrate This method is based on the formation of a colored compound by the reaction of guanidine with *1 2* naphtha quinone-*4*-sodium sulphonate After testing a large variety of compounds Sullivan found this test to have a rather high degree of specificity for guanidine Even the closely related methyl guanidine fails to give the reaction Of the substances likely to be present in urine only ammonia and methyl amine may contribute to the color produced by guanidine This makes it important to remove any picrates of these compounds which may be present as a contaminant in the precipitated guanidine The small amounts of methyl amine which may be present are readily lost through solubility of the picrate According to Greenwald (11) the comparative solubilities of guanidine creatinine and ammonium picrates in 100 cc of water at 20°C are 64 184 and 1020 mgm. respectively Thus it is relatively easier to remove ammonia than creatinine by recrystallization of guanidine picrate from water In the colorimetric determination of guanidine by Sullivan s method the presence of small amounts of creatinine does no harm In our procedure the separated guanidine picrate purified by recrystallization was dissolved in water and the color produced by a suitable aliquot of this solution was compared in a colorimeter with the color produced by 0.5 mgm of guanidine With the combination of methods which we used it was found

that equally good recoveries of guanidine were obtained by omitting the treatment of urine with tannic acid and barium hydroxide and proceeding directly to the evaporation of the urine and subsequent extraction with alcohol

Obviously our whole procedure has been adapted to the determination of the rather large amounts of guanidine present in urine following the ingestion of guanidine hydrochloride It has been reported that small amounts of guanidine or methyl guanidine can be isolated from rather large volumes of normal urine (13) (14) In our study we were not concerned with establishing the presence or absence of traces of guanidine in normal urine With the small (75 to 150 cc) samples used for the determinations no guanidine was isolated from normal urine by our procedure These negative controls

TABLE 1

Recoveries of guanidine hydrochloride added to normal urine

NUMBER	VOLUME OF URINE	GUANIDINE ADDED	GUANIDINE DETERMINED	PERCENTAGE RECOVERED	REMARKS
	cc.	mgm	mgm		
1	200	50	46 0	92 0	Tannic acid + $Ba(OH)_2$ treatment
2	200	50	47 3	94 6	Tannic acid + $Ba(OH)_2$ treatment
3	200	50	48 6	97 2	Tannic acid + $Ba(OH)_2$ treatment
4	200	50	52 7	105 4	Tannic acid + $Ba(OH)_2$ treatment
5	200	50	50 8	100 2	Tannic acid + $Ba(OH)_2$ treatment
6	200	50	52 3	104 6	Tannic acid + $Ba(OH)_2$ treatment
7	200	50	47 8	95 6	Tannic acid + $Ba(OH)_2$ treatment
8	100	50	47 6	95 2	Shorter process, direct evaporation of urine
9	100	40	36 6	91 6	Shorter process, direct evaporation of urine
10	100	30	30 8	102 0	Shorter process, direct evaporation of urine
11	100	50	46 3	92 6	Shorter process, direct evaporation of urine
12	100	20	20 0	100 0	Shorter process, direct evaporation of urine
13	150	25	25 2	100 8	Shorter process, direct evaporation of urine
14	150	25	25 0	100 0	Shorter process, direct evaporation of urine
15	150	20	20 0	100 0	Shorter process, direct evaporation of urine
16	150	20	19 8	99 0	Shorter process, direct evaporation of urine
17	200	50	47 6	95 2	Allowed to stand 24 hours before analysis
18	200	40	36 6	91 6	Allowed to stand 24 hours before analysis
19	200	30	30 8	102 0	Allowed to stand 48 hours before analysis

also indicate that under the conditions of our experiments no significant error is being introduced in our positive results by contamination with picrates other than guanidine picrate A series of recoveries of guanidine added to normal urine are presented in table 1 Provided the urines were made acid to litmus and preserved with chloroform we did not encounter the difficulty mentioned by Sullivan (10) that satisfactory recoveries cannot be made if urine is allowed to stand for some time after the addition of guanidine before the analysis is started As may be seen in table 1 we obtained essentially the same results after a delay of as long as 48 hours

For the determination of glycocyamine use was made of the color reaction described by Sakakuchi (15) following adsorption of the glycocyamine on *Lloyd's reagent* and subsequent elution with barium hydroxide essentially according to the method described by Weber (16, 17) and modified by Bodansky (18) At first poor recoveries of added

glycocyamine resulted because of difficulties encountered with the step which involves the removal of ammonia and possible small amounts of other interfering substances from the final solution by shaking with permutit When pure solutions of glycocyamine were shaken with certain samples of permutit varying amounts of glycocyamine were adsorbed on the reagent With other samples this loss did not occur Repeated tests under different conditions indicated that the difficulty did not arise from any permanent defect in a given lot of permutit but was associated with the state of hydration of the reagent used. We found that recently washed permutit which had been allowed to become superficially dry at room temperature was particularly likely to adsorb glycocyamine Frequently if such a sample was merely set aside to age for several weeks it could then be used without causing a loss of glycocyamine By drying numerous lots of permutit to a constant weight at a high temperature we found that samples which adsorbed glycocyamine contained 30 to 40 per cent of water while those which caused no loss contained only 15 to 20 per cent of water We then found that by drying with heat we could promptly make freshly washed permutit suitable for use We have no explanation to offer as to why permutit in a greater state of hydration should adsorb glycocyamine more readily than a sample of the same reagent from which all but a small percentage of water has geen removed Our experiences however emphasize the necessity of testing each lot of permutit with standard solutions before it is adopted for use with unknown solutions Before we considered a sample of permutit suitable for use in our determinations it had to meet the triple requirement of quantitatively removing arginine and ammonia from a solution and of causing no loss of glycocyamine when shaken with a standard solution of this substance

At times before we recognized what variable was responsible for our difficulties we found ourselves without a satisfactory lot of permutit available for use. On such occasions we resorted to the procedure of removing ammonia by aeration of an alkaline solution Fortunately the first step in carrying out the Sakakuchi color test is to alkalinize the glycocyamine solution by the addition of 1 cc of 10 per cent sodium hydroxide to 5 cc of solution By a series of tests using standard solutions of glycocyamine and also solutions containing both glycocyamine and ammonium salts it was found that the ammonia could be removed leaving the glycocyamine unchanged by aeration of the alkalinized solution for 30 minutes in an apparatus similar to that described by Folin and Farmer (19) Precautions were taken to avoid changes in the concentration of the solution either by the introduction of water or by loss through evaporation during the aeration process In some of the urinary studies presented in this paper the aeration process was used in other permutit which met the criteria outlined above was employed. Both methods gave essentially the same results. On theoretical grounds however the permutit method is preferable since traces of non-volatile interfering substances, such as arginine or methyl guanidine would be removed by permutit but not by aeration

In brief our adopted procedure was as follows

Five or 10 cc acidified urine were shaken with 1 gram of Lloyd s reagent and the adsorbed glycocyamine subsequently eluted by shaking with 7 cc. of a saturated solution of barium hydroxide The barium hydroxide solution containing the liberated glycocyamine was combined with successive small portions of wash water acidified with sulphuric acid and made to a volume of 25 or 50 cc After making this solution alkaline to congo red by the addition of solid basic lead carbonate it was centrifuged and the clear supernatant fluid treated by one of the two methods described above for the removal of ammonia Color was then developed in 5 cc. aliquots of the ammonia free solution by the Sakakuchi reaction according to the procedure described in detail by Weber (16) The colors produced in the unknown solutions were read in a colorime-

ter against the color similarly produced in a standard solution of glycocyamine The standards usually required by the aliquots of urine used in our procedure were solutions containing either 0 025 or 0 050 mgm of glycocyamine in 5 cc Duplicate samples were always carried through the entire procedure The results of a small series of recoveries of glycocyamine from pure solution and in urine by the procedures we have outlined are collected in table 2

Creatine and creatinine were determined by the method of Folin (20) using recrystallized picric acid (21)

TABLE 2

Recoveries of glycocyamine from pure solutions and from urine by various procedures described in paper

NATURE OF MATERIAL ANALYZED	GLYCOCYAMINE			REMARKS
	Mgm. present in portion analyzed	Mgm determined in portion analyzed	Per cent recovered	
Pure solution	0 25	0 23	92 0	Carried through entire procedure
Pure solution	0 25	0 24	96 0	Carried through entire procedure
Pure solution	0 25	0 23	92 0	Carried through entire procedure
Glycocyamine solution + ammonium sulphate	0 50	0 48	96 0	Aeration method
Glycocyamine solution + ammonium sulphate	0 20	0 20	100 0	Permutit method
Urine A		0 38		Average of four determinations
Urine A + glycocyamine	0 48	0 48	100 0	"Amount present" = 0 38 plus 0 10 mgm added
Urine A + glycocyamine	0 48	0 47	98 0	"Amount present" = 0 38 plus 0 10 mgm added
Urine A + glycocyamine	0 58	0 56	94 0	"Amount present" = 0 38 plus 0 20 mgm added
Urine B		0 24		Aeration method
Urine B		0 25		Permutit method
Urine C		0 27		Aeration method
Urine C		0 27		Permutit method
Urine D (5 cc sample)		0 42		Aeration method
Urine D (10 cc sample)		0 81		Aeration method

The experimental subjects were two normal women and three patients with rather severe myasthenia gravis Two of these patients (M O and W M G —cases 2 and 5 of our earlier paper (1)) had been receiving guanidine medication for several months before our studies were carried out The third patient (E A) was a man who had been on prostigmine medication (18 tablets of 15 mgm each daily) for about a month He had never received guanidine medication until the present studies were made All the experi-

mental subjects were placed on a creatinine-free diet. The urine was collected in 24 hour specimens in bottles containing a small amount of chloroform as preservative. All the chemical studies were made on the day that the

TABLE 3

Urinary excretion of ingested guanidine hydrochloride

EXPERIMENTAL SUBJECT	DOSE OF GUANIDINE HCl	CALCULATED DOSE OF GUANIDINE	VOLUME OF URINE	PREFORMED CREATININE	CREATINE	GLYCOCYAMINE	GUANIDINE IN URINE	PER CENT OF DAILY DOSE IN URINE
	mgm.	mgm.	cc.	grams	gram	mgm.	mgm.	
Normal subject, M. K., weight 49 kgm.	0	0	1180	1.22	0.00	20	0	
	500	309	1200	1.24	0.00	24	175	57
	500	309	1000	1.16	0.00	17	188	61
	500	309	1220	1.15	0.00	17	234	76
	500	309	1550	1.10	0.00	19	262	85
	0	0	1400	1.24	0.00	22	98	
	0	0	1200	1.15	0.00	18	87	
Normal subject, A. M. weight 67 kgm.	0	0	1100	1.23	0.02	61	0	
	0	0	1170	1.19	0.00	82	0	
	0	0	1600	1.25	0.00	61	0	
	625	386	2000	1.22	0.00	58	183	47
	375	232	2190	1.17	0.00	57	197	85
	625	386	1100	1.22	0.00	75	195	51
	0	0	1000	1.21	0.00	56	120	
Myasthenia gravis M. O weight 75 kgm. (had taken guanidine at this rate for about a year)	1375	850	2180	1.20	0.00	83	491	58
	1375	850	2080	1.23	0.00	33	501	59
	1375	850	1790	1.32	0.00	25	553	65
	1375	850	1920	1.30	0.00	26	508	60
Myasthenia gravis, W. G. M. weight 40 kgm (had taken guanidine at this rate about six months)	1375	850	1720	0.76	0.00	24	413	49
	1375	850	1940	0.79	0.00	50	454	53
	1375	850	1580	0.60	0.00	25	405	48
	1375	850	2040	0.72	0.00	31	428	50
Myasthenia gravis, E. A., weight 70 kgm. (starting guanidine medication for the first time)	0	0	1000	1.16	0.00	18	None	
	0	0	500	0.73	0.00	13	None	
	1500	936	1010	1.02	0.00	17	299	32
	1500	936	800	0.84	0.06	13	364	39
	1875	1172	800	0.83	0.00	13	405	35
	2250	1406	900	0.84	0.00	13	577	41
	2625	1641	1000	0.91	0.00	15	740	45

24 hour collection was completed. The two older patients continued to take their regular required and well tolerated doses throughout the experimental period. The third patient took varying and increasing doses as we attempted to determine his tolerance and his most satisfactory maintenance dose of the drug. The two normal subjects took the smaller daily doses of guanidine

which they could tolerate without the production of significant symptoms of intoxication The data obtained are presented in table 3

DISCUSSION OF EXPERIMENTAL RESULTS

It is evident from our data that the greater tolerance of patients for guanidine hydrochloride cannot be explained on the basis of a more rapid or complete excretion of the drug than that observed in normal individuals Roughly similar percentages (usually between 40 and 60 per cent) of the daily dose were excreted by all the experimental subjects From this small series of observations one gets the impression that even larger percentages of the ingested guanidine remain unaccounted for in patients than in the two normal individuals This tendency, which might well not be borne out by more extended studies, is most strongly suggested in the patient E H who was receiving guanidine for the first time Regardless of the possible significance of any difference in the percentage excreted, if one considers the daily doses of guanidine ingested a far greater total amount remains unaccounted for in the patients than in the normal women When it is further considered that two of these patients had taken these same daily doses of guanidine for months before the urinary studies were made and have continued to take similar amounts for months since that time, their ability to do something with guanidine other than to excrete it unchanged becomes even more impressive

Some guanidine is probably retained for a time unchanged in the body In accord with this assumption is the continued excretion of considerable amounts for a day or two after administration had ceased in the two normal subjects Most of this must have been held in the tissues since no significant elevation of guanidine in the blood resulted from the amounts administered It is, however, inconceivable that storage of unchanged guanidine in the body at this rate could go on for months of continued medication One is forced rather to conclude that much of the dose ingested by patients is being transformed to some other compound

Our studies presented here fail to throw any light on the problem of what substances may be formed from guanidine There was no increase in the urinary output of creatine or glycocyamine following guanidine administration None of the subjects had any significant creatinuria at any time during the observation period The figures for glycocyamine are in the range reported by Weber (22, 23) for normal adults and are in accord with his finding that women have a somewhat larger daily excretion of this constituent than men Our negative results are in no sense a proof that guanidine may not have been transformed into glycocyamine and creatine and retained in the body The data presented simply show that guanidine not excreted unchanged does not promptly appear in the urine as either creatine or glycocyamine

SUMMARY AND CONCLUSIONS

Procedures have been described which give satisfactory recoveries of guanidine and glycocyamine (guanido acetic acid) added to urine.

Studies of the urinary excretion of guanidine, glycocyamine, creatine and creatinine of three patients with myasthenia gravis treated with guanidine hydrochloride and of two normal women receiving smaller daily doses of the same drug lead to the following conclusions

The greater tolerance of persons with myasthenia gravis for guanidine hydrochloride cannot be explained on the simple basis of a more rapid excretion of the drug Both patients and controls excrete in the neighborhood of 40 to 60 per cent of the daily ingested dose of guanidine unchanged in the urine

The ingestion of tolerated doses of guanidine does not lead to increased urinary excretion of either creatine or glycocyamine by normal persons or by patients with myasthenia gravis.

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OBSERVATIONS ON THE TOXICOLOGY OF SULFATHIAZOLE AND SOME RELATED COMPOUNDS

H A WALKER AND H B VAN DYKE

From the Division of Pharmacology, The Squibb Institute for Medical Research, New Brunswick, N J

Received for publication November 9, 1940

The toxicology of sulfathiazole, 2(*p*-amino-benzene-sulfonamido) thiazole, has been investigated both in laboratory animals and in man in view of its probable importance in the treatment of lymphogranuloma venereum, gonorrhea, and infections of the genito-urinary tract, as well as those caused by pneumococci, staphylococci, and streptococci These observations have been extended in the present communication to include comparisons of sulfathiazole with analogues such as sulfapyridine and sulfamethylthiazole with respect to acute lethal action, chronic toxic effects, and behavior after absorption from the gastro-intestinal tract has occurred In addition, the acute lethal action of sulfanilamide, sulfaethylthiazole, and sulfaphenylthiazole has been determined

A COMPARISON OF THE MEDIAN LETHAL DOSES OF SULFANILAMIDE, SULFATHIAZOLE, AND ANALOGUES OF SULFATHIAZOLE

A considerable number of experiments was performed to determine the acute lethal effects of a single dose of sulfanilamide, sulfathiazole, or derivatives of sulfathiazole administered subcutaneously as the soluble sodium salt to Swiss mice The data of these acute toxicity experiments are summarized in table 1 In the table, the dosages of each drug in terms of the free acids have been arranged in an ascending order The order in which the experiments were performed is shown by the order of the lettering of the experimental groups Thus, in a given experimental group, two to four drugs were tested with a large homogeneous batch of mice and each drug was given at one, two, or in some instances three dose levels For example, in Experiment E sulfathiazole and its three derivatives were each tested at three dose levels [1] Although there was rarely any death after 48 hours, all observations were completed only after 72 hours had elapsed The most erratic results were obtained in groups of mice receiving sulfaphenylthiazole Na, from which recovery might or might not slowly take place after a long period of coma

[1] Only the lowest dose causing 100 per cent mortality is recorded in table 1 Therefore all results of Experiment E, for example, will not be found in this table

TABLE 1

Toxicity of sulfanilamide sulfathiazole or derivatives of sulfathiazole administered as a single subcutaneous dose of the sodium salt to Swiss mice

EXPERIMENT GROUP	NUMBER OF MICE	DRUG	DOSE*	MORTALITY	CALCULATED L.D.50* AND S.E. OF L.D.50
			grams per kgm.	*per cent*	*grams per kgm.*
C	25	Sulfathiazole Na	1.00	24	
E	20	Sulfathiazole Na	1.00	20	
B	25	Sulfathiazole Na	1.25	60	
D	25	Sulfathiazole Na	1.25	40	
E	20	Sulfathiazole Na	1.33	35	
F	20	Sulfathiazole Na	1.38	50	1.320 ±0.035
G	20	Sulfathiazole Na	1.40	50	
H	20	Sulfathiazole Na	1.40	55	
A	25	Sulfathiazole Na	1.50	72	
E	20	Sulfathiazole Na	1.67	70	
F	20	Sulfathiazole Na	1.67	80	
E	20	Sulfamethylthiazole Na	0.69	5	
C	25	Sulfamethylthiazole Na	0.79	32	
D	25	Sulfamethylthiazole Na	0.90	36	0.885 ±0.021
E	20	Sulfamethylthiazole Na	0.95	75	
E	20	Sulfamethylthiazole Na	1.21	100	
F	20	Sulfaethylthiazole Na	0.17	5	
G	20	Sulfaethylthiazole Na	0.20	25	
E	20	Sulfaethylthiazole Na	0.22	60	0.226 ±0.012
G	20	Sulfaethylthiazole Na	0.27	85	
F	20	Sulfaethylthiazole Na	0.28	75	
G	20	Sulfaethylthiazole Na	0.33	100	
E	20	Sulfaphenylthiazole Na	0.26	0	
D	25	Sulfaphenylthiazole Na	0.32	8	
F	20	Sulfaphenylthiazole Na	0.39	5	
G	20	Sulfaphenylthiazole Na	0.39	25	0.462 ±0.035
F	20	Sulfaphenylthiazole Na	0.49	15	
E	20	Sulfaphenylthiazole Na	0.52	90	
G	20	Sulfaphenylthiazole Na	0.52	95	
G	20	Sulfaphenylthiazole Na	0.65	100	
H	20	Sulfanilamide Na	2.20	35	
H	20	Sulfanilamide Na	2.40	60	2.314 ±0.044
H	20	Sulfanilamide Na	2.70	90	
	23	Sulfapyridine Na	0.50	4	
	30	Sulfapyridine Na	0.75	17	0.807† ±0.079
	30	Sulfapyridine Na	1.00	97	

* As free acids.

† Calculated from the data of van Dyke, Greep Rake and McKee (2)

The last column of table 1 shows the median lethal doses (L D 50) of the drugs calculated as free acids and the standard errors of these doses Both values were estimated by the method of Bliss (1) Sulfathiazole Na, while by these tests much more toxic than sulfanilamide Na, is considerably less toxic than sulfapyridine Na or the other sulfathiazole analogues The acute lethal action of sulfamethylthiazole Na is not significantly different from that of sulfapyridine Na Of particular interest is the unexpectedly high toxicity of sulfaethylthiazole Na in comparison with the methyl and phenyl derivatives For a comparison of these drugs on a molecular basis, the median lethal doses of the Na salts are expressed in figure 1 in millimols per kilogram of body weight of mouse The standard errors of these values similarly expressed can be calculated from table 1

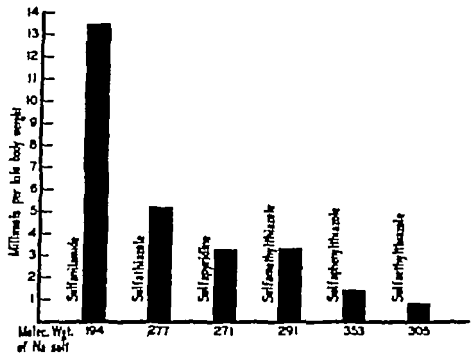

FIG 1 MEDIAN LETHAL DOSES (L D 50) OF VARIOUS SULFONAMIDES IN MICE BASED UPON THE RESULTS SUMMARIZED IN TABLE 1

THE EFFECTS OF LONG-CONTINUED ADMINISTRATION OF SULFATHIAZOLE, SULFAMETHYLTHIAZOLE, OR SULFAPYRIDINE

The effects of repeated doses of sulfathiazole, sulfamethylthiazole, and sulfapyridine as the free acids were studied in rats Groups of young rats (Long-Evans strain, 7 males and 7 females in each group) were fed diets containing 0 5 or 1 0 per cent of the drugs for a period of 50 days The consumption of food and drug was not measured throughout the entire experiment, but was determined twice at an interval of five weeks for periods of three days each After the rats had been on the diets for 10 days and were then 40 days old, the average amounts of drug eaten per kilogram body weight per 24 hours were 0 61 grams for 0 5 per cent sulfathiazole, 1 17 grams for 1 per cent sulfathiazole, 0 55 grams for 0 5 per cent sulfapyridine, 1 18 grams for 1 per cent sulfapyridine, 0 61 grams for 0 5 per cent sulfamethylthiazole, and 1 23 grams for 1 per cent sulfamethylthiazole Five weeks

later the consumptions of drug, expressed as above, were 0 45 grams for 0.5 per cent sulfathiazole 1 03 grams for 1 per cent sulfathiazole, 0 43 grams for 0.5 per cent sulfapyridine, 0 95 grams for 1 per cent sulfapyridine, and 0.52 grams for 0 5 per cent sulfamethylthiazole.[3]

The weight curves of the male animals are shown in figure 2 and demonstrate distinctly that at the 1 0 per cent level sulfathiazole was the least toxic and sulfamethylthiazole the most toxic of these drugs. Although at

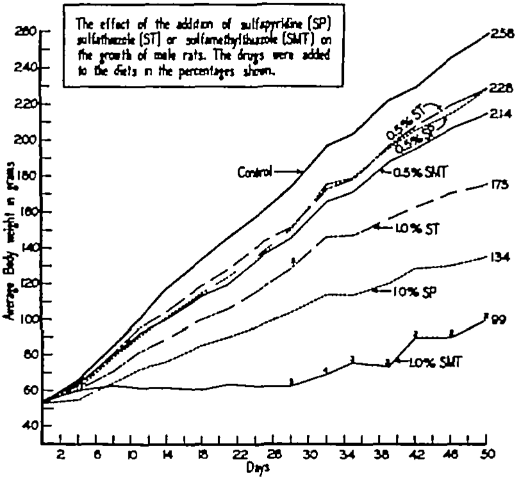

FIG 2. SEE ALSO TEXT

this level sulfapyridine was much more toxic than sulfathiazole there was no difference between these two at the 0.5 per cent level but sulfamethyl thiazole possibly was again the most toxic In our first report (2) a figure illustrated the effects of the addition of 0 5 or 1 0 per cent sulfathiazole or sulfapyridine on the growth curves of female rats Experiments in male rats while yielding comparable results, were not satisfactory because a sudden unexplained loss of weight in control animals and in those receiving sulfa

[3] No value for 1 per cent sulfamethylthiazole is given here since there was great variation in food consumption among the three animals surviving from a group of 14

thiazole occurred in the middle of the experimental period The results obtained in similar experiments repeated in female rats were like those first reported in that the rate of growth of female rats receiving 0 5 per cent sulfathiazole was only slightly lower than that of the controls and the growth rate of female animals receiving 1 0 per cent sulfathiazole was similar to that of females on a diet of 0 5 per cent sulfapyridine (or 0 5 per cent sulfamethylthiazole) The slowest growth rate of female rats was observed when 1 0 per cent sulfamethylthiazole was fed It is possible that in growing male rats on a diet containing 0 5 per cent of drug, sulfathiazole is more toxic than in growing females At the 1 0 per cent level in either sex, the order of deleterious effect on growth is sulfamethylthiazole > sulfapyridine > sulfathiazole

No deaths among the male or female animals occurred at the 0 5 per cent level However, at the higher level only 1 of 7 female and 2 of 7 male animals receiving sulfamethylthiazole survived, whereas no deaths attributable to the drugs occurred in either the sulfathiazole or sulfapyridine groups (A few animals died acutely when heart punctures were performed to secure blood for quantitative analyses of drugs, this occurred in 2 male rats receiving the 1 per cent sulfathiazole diet)

At the end of the experiment in which the chronic toxic effects of the three drugs were studied, all the rats were killed after blood had been removed for estimating the concentration of drugs present and for making blood smears The gross appearance of the important organs was examined and the only important pathological changes were found in the kidneys, ureters, and bladder and in the spleen. Portions of the liver, kidneys, and spleen of each animal were fixed for microscopic study

No gross or microscopic changes which could be attributed to drug were found in the livers of any of the experimental rats The spleens of all animals were normal except those receiving the diet containing 1 0 per cent sulfamethylthiazole Only three of these rats survived the whole experimental period and in these the spleens were normal. In all the others the changes resembled those found in mice receiving a diet containing sulfathiazole (3) The spleens were commonly atrophic Microscopically there was a reduction or disappearance of the germinal centers of the malpighian bodies with an accompanying loss of lymphocytes from the mantle and marginal zones Occasionally the cells of the red pulp also disappeared and little but supporting tissue remained

Pathological changes in the kidneys or concretions in the ureters or bladder, either separately or together, were found in all groups except control animals The frequency of concretions was highest in rats receiving sulfamethylthiazole (94 per cent of 16 rats), intermediate in the rats on sulfapyridine (43 per cent of 28 rats), and lowest in rats on sulfathiazole (28 per cent of 24 rats)[3] There was a significantly greater incidence of concretions in rats receiving sulfamethylthiazole than in those on diets containing the other drugs, among the latter there was no statistically significant difference in incidence The frequency of such lesions was the same whether the diet contained 0 5 per cent or 1 0 per cent of drug Pathological changes in the kidney were

[3] All rats on which necropsies could be performed, including those not surviving until the end of the experiment, are listed

rarely present unless associated with grossly visible concretions in the kidneys, ureters or bladder (The exceptions were one rat receiving 1 0 per cent sulfathiazole diet and another rat receiving 1.0 per cent sulfapyridine diet) On the other hand, among 34 animals in which concretions were found, renal lesions were present in 25 but absent in 9 The principal renal abnormalities were hydronephrosis with varying degrees of destruction of the renal parenchyma, tubular dilatation the presence of casts cellular débris or rarely of blood in the tubules, and areas of leukocytic infiltration about some dilated tubules which appeared once to have contained crystalline material

Differential white cell counts were made on all animals at the conclusion of the experiment and little or no difference in the blood picture among the animals receiving sulfathiazole, sulfapyridine and sulfamethylthiazole at the 0.5 per cent level was found. The distribution of leukocytes in the blood of animals receiving sulfathiazole at a 1 0 per cent level in the diet was likewise similar to that of normal rats In rats on a 1 0 per cent sulfapyridine diet the percentage of neutrophiles was 27 6 per cent (normal 17.1 per cent) and the percentage of lymphocytes was 70 4 per cent (normal, 81 0 per cent) A more marked change in the same direction was observed in rats on a diet of 1.0 per cent sulfamethylthiazole. Of the total leukocytes 44.3 per cent were neutrophiles and only 54.0 per cent were lymphocytes. Since circumstances did not permit the making of counts of the number of leukocytes per cmm. of blood it is not possible to state whether the observed changes were in the proportion or in the absolute number of neutrophiles or lymphocytes In our earlier experiments (3) no effects of sulfathiazole or sulfapyridine on the blood counts including differential counts, could be demonstrated.

OBSERVATIONS ON THE METABOLISM OF SULFATHIAZOLE, SULFAMETHYLTHIAZOLE, OR SULFAPYRIDINE

Observations comparing the metabolism of sulfapyridine and sulfathiazole were recorded in our first report (2) These studies indicated that sulfathiazole is more rapidly metabolized and undergoes less conjugation than sulfapyridine. Further data on blood levels and urinary excretion after the oral administration of sulfathiazole sulfamethylthiazole, or sulfapyridine to rats have been gathered and are presented here The determinations were made by the method of Bratton and Marshall (4) A Pulfrich photometer was used for the final estimations in which filter S55 was employed Standardization curves of each drug were made from pure samples

In the course of the experiment testing the toxic effects of the three drugs incorporated in the food on the rate of growth of rats, it was planned to secure quantitative estimates of drugs in the blood of individual rats bled simultaneously from the heart at different hours in the day It was anticipated that variations might be considerable and if large, would be related to diurnal periods of greatest food consumption Accordingly determinations of the food consumption of adult rats similar to the experiments of Litchfield, White, and Marshall (5) in mice were made without however mixing any drug with the food. Six rats were kept in individual cages permitting the quantitative determination of the consumption of the stock diet.[4] Consistent results

[4] The stock-diet used consisted of yellow corn, 30 per cent whole wheat, 29 per cent whole milk powder 21 per cent meat scrap 10 per cent linseed meal 7 per cent alfalfa, 2 per cent cod liver oil 1 per cent.

were not obtained until the third day, careful measurements were then made for 4 days The results, which are illustrated in figure 3, represent the mean food consumption of 24 individual twenty-four hour periods Our results in rats resemble those of Litchfield, White, and Marshall in mice in showing that both rodents consume the greater part of their food at night and eat least in the forenoon However, the maximum rate of food consumption in rats was found in the periods 8 p m to midnight and midnight to 4 a m, whereas in mice (5) the maximum rate was considered to fall between 9 p m and 1 a m

Blood was secured at various hours of the day and night from the heart of rats receiving the diet containing a drug in the chronic intoxication experiment Because of an occasional death owing to the puncture and because

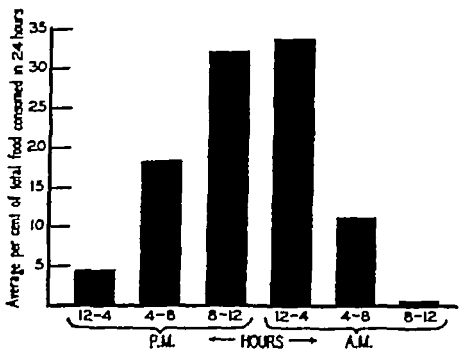

FIG 3 THE CONSUMPTION OF FOOD BY RATS AS MEASURED AT INTERVALS OF 4 HOURS

of the poor condition of certain animals (e g, rats on a diet of 1 per cent sulfamethylthiazole), attempts to gather adequate data were abandoned However, what results were obtained (six individual determinations during a twenty-four hour day in each of the 6 groups) suggested that *1*, peak blood-levels would be found generally between 11 p m and 9 a m and *2*, the blood-concentration of sulfathiazole, at a given drug level in the diet, would be the lowest whereas that of sulfamethylthiazole would equal or excel that of sulfapyridine Minimum levels of free drug in blood commonly occurred at 4 p m but might be present as early as 11 a m (Between 11 a m and 4 p m no blood was collected)

Each rat was also bled before necropsy was performed at the end of the experiment designed to test the chronic toxic effects of the drugs, and the concentration of free and conjugated drug in the blood was determined The results are summarized in table 2 The blood samples were all collected between 10 and 11 30 a m, at a time when the concentrations of the drugs

should be falling Comparing the mean concentrations of the drugs in the blood at the 0 5 and 1 0 per cent levels in the food one may see that the concentration of free or conjugated sulfapyridine was about twice that of sulfathiazole and that the concentration of free sulfamethylthiazole was significantly higher than that of free sulfapyridine (We have no explanation to offer for the low level of conjugated sulfamethylthiazole in rats on the 0 5 per cent diet.) The lower blood-levels of sulfathiazole in these rats suggests that either *1* sulfathiazole is excreted at a more rapid rate or *2* this drug is absorbed less adequately than the other two The fact that the proportion of sulfathiazole in conjugated form resembles that of sulfapyridine at comparable blood levels suggests that either (*A*) when the blood level is falling, this occurs more rapidly for free sulfathiazole than for free sulfa

TABLE 2

The terminal concentration of free and conjugated drug in the blood of rats of both sexes consuming food containing drug for 50 days

DRUG	PER CENT IN FOOD	NUMBER OF RATS	MEAN AND S.E. OF MEAN CONCENTRATION OF DRUG IN BLOOD	
			Free	Conjugated
			mgm. per cent	*mgm. per cent*
Sulfapyridine	0 5	14	4 36 ±0 52	2 62 ±0 19
Sulfapyridine	1 0	14	9 81 ±0 62	4 71 ±0 31
Sulfathiazole	0 5	14	2 37 ±0 23	1 29 ±0 17
Sulfathiazole	1 0	11	4 36 ±0 42	2 46 ±0 30
Sulfamethylthiazole	0 5	8	5 53 ±0 44	0 90 ±0 27
Sulfamethylthiazole	1 0	3	10 60 ±0 25	4 60 ±0 50

pyridine and, consequently, more of the less readily excreted conjugated sulfathiazole is found in the blood than would be the case earlier or (*B*) there is no important difference in the degrees to which sulfathiazole and sulfa pyridine undergo conjugation. Hypothesis *1* accords with the overwhelming bulk of available data. Hypothesis (*A*) would explain the generally accepted conclusion that in mammals which conjugate sulfonamides, with the exception of the rabbit receiving the drug orally sulfapyridine is usually conjugated to a greater extent than sulfathiazole.

To extend the observations on the metabolism of sulfathiazole and sulfa pyridine 5 rats each received 0.513 or 0 500 gram per kilogram of free acid suspended in gum acacia solution as a single daily dose by stomach tube for 10 days. The rats, of the Long Evans strain were all adult males. They were kept in individual metabolism cages on the stock-diet. Any animals in which diarrhea appeared were excluded. The urine was carefully collected each day and the total daily excretion of free and conjugated drug was deter mined Typical observations in two of the rats are shown in figure 4 All

the data are summarized in table 3 A total of 50 twenty-four hour periods is represented by each group Averages for individual rats were first determined and the mean of these averages was calculated as a basis for estimating the standard errors recorded in table 3 If Fisher's well known method of paired comparisons is used to calculate the probability that as great varia-

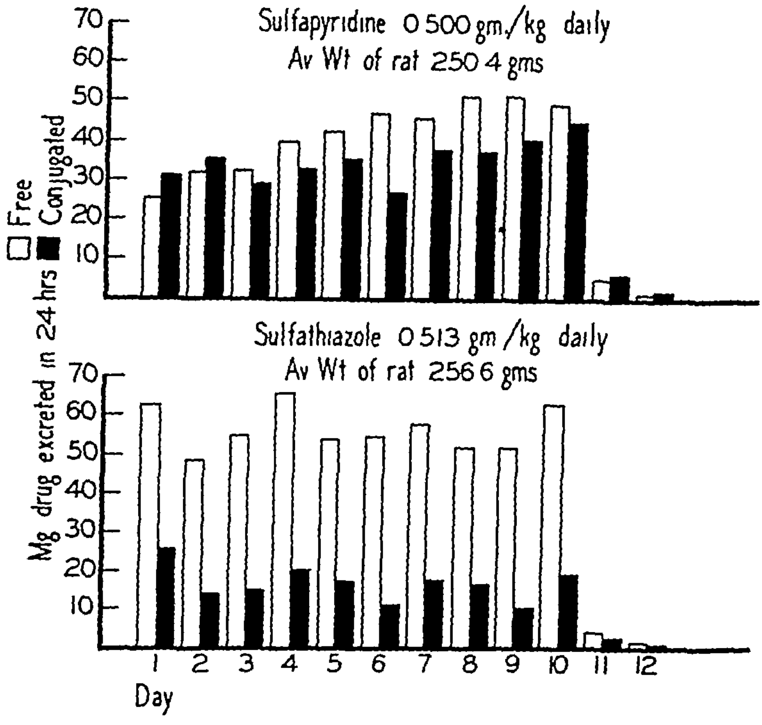

FIG 4 THE TOTAL URINARY EXCRETION OF FREE AND CONJUGATED DRUGS COMPARED IN TYPICAL EXPERIMENTS IN INDIVIDUAL RATS

The drugs were administered once daily by stomach tube The first dose was given when the collection of the 24 hour urine of day 1 was begun and the last dose when the collection of urine on day 10 was begun

tions in the excretions of either free or conjugated drug might occur by chance, the probability in both instances is less than 0 01 From these data it appears warranted to conclude that (*I*) over a period of 24 hours approximately equal total amounts of single doses of sulfathiazole or sulfapyridine are absorbed by rats if the kidneys are considered to be the *only important channel* of excretion and (*II*) about twice as much sulfapyridine as sulfathiazole undergoes conjugation These experimental results do not contradict hypothesis *1* and agree with hypothesis (*A*)

A few experiments were also performed in rabbits in an attempt to compare sulfapyridine and sulfathiazole when the blood-levels of the drugs were comparable. An aqueous suspension of sulfapyridine or sulfathiazole was injected intraperitoneally into 2 rabbits daily for 10 days. The dose of sulfapyridine given once daily was 0.75 to 1.0 grams per kilogram, while that of sulfathiazole was 1.0 to 1.25 grams per kilogram. The averages of 28 blood concentrations revealed little difference between the 2 drugs in total amount of drug but the amount of conjugated sulfapyridine was 66 per cent of the total (9.08 mgm. per cent total) as compared to 51 per cent for conjugated sulfathiazole (9.06 mgm. per cent total drug in blood). In a similar experiment with 8 rabbits receiving sulfapyridine or sulfathiazole at doses of 1.0 and 1.1 grams per kilo for 3 days the amount of conjugated drug in the blood was found to be 59 per cent of the total sulfapyridine and 28 per cent of the total sulfathiazole in the blood. In the latter experiment the total amount

TABLE 3

The excretion of sulfapyridine and sulfathiazole by rats to which daily oral doses were administered for 10 days

DRUG	NUMBER OF RATS	AVERAGE AND S.E. OF WEIGHT OF RATS	AVERAGE DAILY DOSE	AVERAGE AND S.E. OF DAILY TOTAL EXCRETION	AVERAGE AND S.E. OF PER CENT OF DOSE EXCRETED DAILY	AVERAGE AND S.E. OF PER CENT OF TOTAL DRUG EXCRETED DAILY IN CONJUGATED FORM
		grams	*mgm.*	*mgm.*		
Sulfapyridine	5	248.3 ±8.0	121.7	67.5 ±6.1	55.4 ±5.0	44.9 ±4.6
Sulfathiazole	5	251.8 ±8.9	129.0	70.5 ±2.2	54.6 ±1.7	20.9 ±1.6

of sulfapyridine in the blood (13.4 mgm. per cent) was 8 per cent greater than that of sulfathiazole. Toxic symptoms appear in rabbits when high levels of conjugated sulfathiazole (35 to 45 mgm. per cent) were found in the blood. These high levels of conjugated drug were not included in the averages given above and were followed a day or two later, by the death of the rabbits. Of the 5 rabbits which received sulfathiazole there was one survival, while with sulfapyridine there was only one death. The toxic symptoms were similar to those reported by Marshall, Bratton, White and Litchfield (6).

DISCUSSION

Marshall, Bratton and Litchfield (7) clearly showed that serious errors in measurements of the acute toxicity of sulfonamides may be introduced by failure to ensure absorption, as when the free acid of a drug like sulfapyridine is administered by stomach tube. Accordingly in all determinations of acute lethal action the soluble sodium salts were injected subcutaneously. The data offered in this report confirm the earlier findings in this laboratory and

the data are summarized in table 3 A total of 50 twenty-four hour periods is represented by each group Averages for individual rats were first determined and the mean of these averages was calculated as a basis for estimating the standard errors recorded in table 3 If Fisher's well known method of paired comparisons is used to calculate the probability that as great varia-

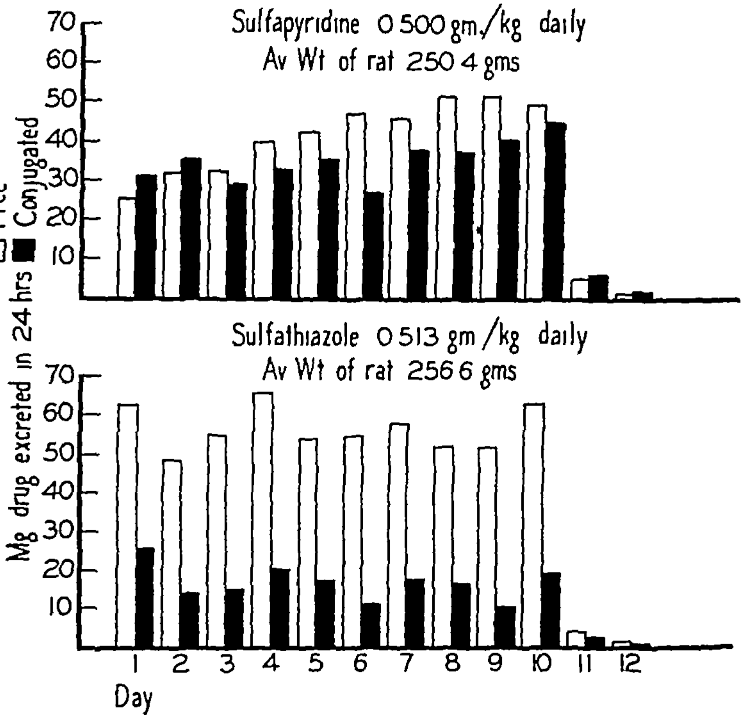

FIG 4 THE TOTAL URINARY EXCRETION OF FREE AND CONJUGATED DRUGS COMPARED IN TYPICAL EXPERIMENTS IN INDIVIDUAL RATS

The drugs were administered once daily by stomach tube The first dose was given when the collection of the 24 hour urine of day 1 was begun and the last dose when the collection of urine on day 10 was begun

tions in the excretions of either free or conjugated drug might occur by chance, the probability in both instances is less than 0 01 From these data it appears warranted to conclude that (*I*) over a period of 24 hours approximately equal total amounts of single doses of sulfathiazole or sulfapyridine are absorbed by rats if the kidneys are considered to be the only important channel of excretion and (*II*) about twice as much sulfapyridine as sulfathiazole undergoes conjugation These experimental results do not contradict hypothesis *I* and agree with hypothesis (*A*)

the mouse, monkey, and man (2, 8, 22, 23, 24)[5] The excretion of sulfathiazole likewise takes place at a more rapid rate than that of sulfapyridine However, in our experiments with rats the urine was collected only once in 24 hours thus obscuring a probable difference in the rate of absorption and excretion of the two drugs which were administered only once daily by stomach tube over a period of 10 days It was found that the twenty four hour excretion of the two drugs was equal but that the proportion excreted in conjugated form was much lower in the case of sulfathiazole. Therefore it appears that the rate of removal of free sulfathiazole from the body is greater than that of sulfapyridine and that less of it undergoes conjugation. With long-continued administration of similarly distributed doses the blood level will be lower dose for dose in comparison with sulfapyridine. That the drug is conjugated to a less extent than sulfapyridine may not be reflected in the levels of free and conjugated drug in the blood

SUMMARY

The doses of the sodium salts of sulfanilamide, sulfapyridine sulfathiazole, and certain analogues of sulfathiazole which after a single subcutaneous injection into albino Swiss mice cause a mortality of 50 per cent, were, in terms of millimols per kilogram body weight, as follows sulfanilamide 13 45, sulfapyridine, 3.24 sulfathiazole 5 18 sulfamethylthiazole, 3.29, sulfaethylthiazole, 0.80 sulfaphenylthiazole, 1.39

The toxic effects of repeated doses of sulfapyridine sulfathiazole and sulfamethylthiazole were compared by feeding each drug mixed with the food in concentrations of 0.5 and 1 0 per cent to groups of growing male and female rats for 50 days. The rate of growth, the effects on the liver kidneys, spleen, and leukocyte-distribution as well as the terminal blood-levels were all determined. Concretions and associated damage of the kidneys an increase in the percentage of neutrophiles in the blood, as well as interference with growth, were all most strikingly present as a result of the administration of sulfamethylthiazole. Only sulfamethylthiazole caused lymphoid atrophy of the spleen in rats Sulfathiazole caused no change in the distribution of leukocytes. In some mammals such as the mouse and rabbit, toxic symptoms and accompanying pathological changes are more pronounced after the oral administration of repeated doses of sulfathiazole than after sulfapyridine similarly administered (2 3 6)

[5] Marshall Bratton White and Litchfield (6) have observed that after oral administration to rabbits the proportion of conjugated drug in the blood was greater for sulfathiazole than for sulfapyridine However the total amount of sulfathiazole in the blood was much greater than that of sulfapyridine Our results show that with approximately equivalent amounts of total drug in the blood the proportion of drug conjugated was greater for sulfapyridine

Additional metabolic experiments in rats indicated that after administration by stomach tube, sulfathiazole and sulfapyridine were equally well absorbed during the 24 hours following a single dose but that much less of the sulfathiazole underwent conjugation It is probable that free sulfathiazole is removed from the blood more rapidly than free sulfamethylthiazole or free sulfapyridine whereas conjugated drug is probably differently excreted

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THE ENZYMIC INACTIVATION OF SUBSTITUTED PHENYL-PROPYL-(SYMPATHOMIMETIC)-AMINES

KARL H. BEYER

From the Department of Physiology University of Wisconsin Medical School Madison

Received for publication November 12, 1940

In view of the widespread interest experimental and clinical, in the action and uses of amines related to adrenaline and ephedrine it is of fundamental importance that we have an understanding of how these compounds are inactivated. It is primarily on this basis that we may ultimately explain and possibly anticipate the relative toxicity, duration of action determining factors in route of administration as well as certain side effects of these similar agents.

The purpose of this research has been to investigate the relationship of molecular configuration and substitution to the mode of enzymic inactivation of certain phenylpropylamines.

PROCEDURE

The amines studied in this series were

a. β-phenylpropylamine
b β-phenylisopropylamine (amphetamine)
c γ-phenylpropylamine
d. α-methyl-γ-phenylpropylamine
e. β-phenyl-β-hydroxyisopropanolamine (propadrine)
f β-phenyl-β-hydroxyisopropanol, methylamine (ephedrine)
g β-(4-hydroxyphenyl) isopropylamine (paredrine)
h. β(4-hydroxyphenyl) isopropylmethylamine
i. β(4-hydroxyphenyl)-β-hydroxyisopropanol, methylamine (suprifen)
j β(3 4-dihydroxyphenyl) isopropylamine
k. β(3 4-dihydroxyphenyl)-β-hydroxyisopropanolamine (cobefrin)

The enzymes used were amine oxidase and phenol oxidase.

Several methods have been described for the preparation of amine oxidase (1 2, 3) These differ considerably and as yet no highly active purified preparation has been reported Since we were not as interested in the constitution of the enzyme or enzyme system itself as in its action on these amines a rather simple preparation sufficed for our purposes. The liver from rabbits, rats or guinea pigs was ground with powdered glass and an equal volume of M/4 phosphate buffer pH 7.30 When the mixture was ground to an even consistency 2 volumes more of buffer were added and the brei ground

for 5 to 10 minutes longer This was then filtered through double layers of muslin The glass remaining in the filtrate was allowed to settle for 15 minutes and the remaining suspension removed by decantation This finely suspended extract constituted the enzyme preparation and was used immediately or allowed to stand at 0°C over night

Phenol oxidase has been prepared in a more highly purified state by Keilin and Mann (4) from mushrooms, and by Kubowitz (5) from potatoes Extracts from the potato were used since they seemed to have for our purposes the advantages of the more highly purified extracts plus simplicity of preparation Graubard and Nelson (6) also found the simpler extracts to be as satisfactory for such purposes as the more highly purified enzyme In most of our experiments the potatoes were peeled, ground first in a meat mincer and then in a mortar until finely mashed From the mash the juice was pressed through double layers of muslin This extract was allowed to stand 15 minutes and then filtered by suction The filtrate was allowed to stand over night at 0° before being used, whereupon it retained but a minimum of residual O_2 uptake These extracts retain most of their activity for over 72 hours

The rate of reaction and the oxygen used in the oxidation of the compounds were measured by means of the Warburg apparatus The experiments using the Warburg type of respirometer were in general arranged as follows

1 5 cc	amine oxidase or phenol oxidase
0.3 cc	M/4 buffer
0 2 cc	amine in M/16 buffer
2 0 cc	total volume

In experiments where cyanide was used 0 1 cc of M/25 or M/15 NaCN plus 0 2 cc M/4 buffer were substituted for the 0 3 cc M/4 buffer The amines were all M/16 in terms of the organic ion Air was used as the source of oxygen except in an occasional experiment with the amine oxidase when O_2 was substituted

In some of the experiments slices of liver from young adult animals were used Into the flasks was measured 1 8 cc Ringer phosphate buffer containing 0 2 per cent fresh glucose to which was added 0 2 cc of the amine, M/16 These tests were conducted in the presence of O_2 In all the experiments filter paper saturated with 10 per cent KOH was contained in the well of the flasks to absorb any CO_2 given off

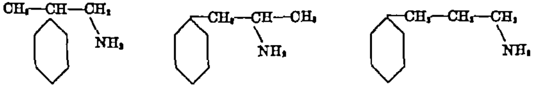
Deamination of the compounds was measured by the following means Two 150 cc test tubes closed by 2 hole rubber stoppers were connected in series by glass tubing This was done in such a manner that air drawn through the tubes could be used to bubble through solutions contained in both tubes The first of the two tubes was clamped submerged to its rim in a water bath designed to hold four units and maintained at 37°C The second tube remained outside the bath The first tube contained the enzyme preparation, amine and buffer, the second contained 40 cc of N/20 HCl Air drawn through the solution in the tube served to oxygenate and agitate the system The NH_3 given off during the reaction was drawn over into the HCl in the second tube The amount of NH_3 dissolved in the HCl was calculated by micro-titrating the excess HCl with NaOH against methyl orange as an indicator The HCl and NaOH solutions were standardized against Na_2CO_3 The bath rested on two electric hot plates which could be used to raise the temperature of the water at will

Experiments were allowed to run for 18 hours at the end of which time 5 cc of 10 per cent NaOH were added to each tube in the bath and the water brought up to 85° for 3 hours to distill over any NH_3 remaining in solution The tests and controls were set up in duplicate The difference in the average titration values for tests and controls was used to calculate the amount of NH_3, attributable to deamination

RESULTS

Amine oxidase

Of the phenylpropylamines tested only those having the amino group on the terminal carbon atom of the side chain were oxidatively deaminated in the presence of amine oxidase This effect seems to be independent of the distance of the amine from the phenyl ring. These two observations may be illustrated by considering the three unsubstituted structural isomers β-phenylpropylamine (a) β-phenylisopropylamine (b), and γ-phenylpropyl amine (c)



(a) (b) (c)

Figure 1 illustrates the rate of reaction and O_2 uptake of (a) and (c) as measured manometrically over a period of 3½ hours using the Warburg respirometer Amphetamine (b) caused no increase in oxygen uptake over that of the controls.

Pugh and Quastel (2) found that the presence of cyanide did not affect the activity of amine oxidase in the oxidation of tyramine or indolethylamine. Earlier Philpot (7) had found that cyanide did not exhibit the oxidation of tyramine by liver and Bernheim (8) observed that in the presence of cyanide the oxidation of tyramine resulted in an uptake of two atoms of oxygen In agreement with these earlier findings amine oxidase did activate the oxidation of both (a) and (c) in the presence of cyanide In preparations low in enzyme content one can best demonstrate activity of the enzyme by inhibiting respiration of the preparation by the use of cyanide

We have found that in the absence of cyanide the QO_2 of liver slices from rabbits, rats and guinea pigs is not significantly altered by the presence of either of these two amines. If cyanide be added to check the normal respiration of the tissue slices, one can demonstrate the activity of the enzyme present in the slices. This is illustrated in figure 2.

That the oxygen uptake resulted in deamination was demonstrated by the method described above. These experiments were set up in duplicate as follows

Controls	*Tests*
25 gram amine oxidase extract from rabbit liver	25 grams amine oxidase preparation
20 cc. phosphate buffer M/20 pH 7.36	75 mgm amine dissolved in 20 cc. phosphate buffer M/20 pH 7.36

The amount of ammonia recovered from three such experiments using 75 mgm of γ-phenylpropylamine sulfate was 7 10, 6 62 and 5 98 mgm This represents recoveries of 103, 96 and 87 per cent of the theoretical amount, 6 9 mgm of NH_3

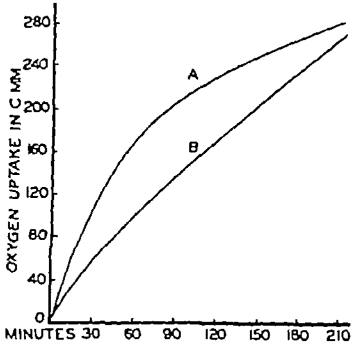


FIG 1 The rate of reaction and oxygen uptake of equimolar concentrations (M/16) of γ-phenylpropylamine (A) and β phenylpropylamine (B) sulfate activated by amine oxidase Buffered at pH 7 36, temperature = 38°C

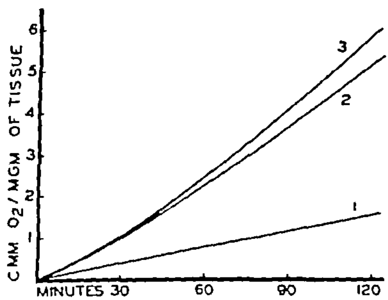


FIG 2 (1) Respiration of guinea pig liver slices (dry weight) in the presence of air and M/250 cyanide (2 and 3) Increase in oxygen uptake of tissue slices in the oxidation of β phenylpropylamine and γ-phenylpropylamine sulfate, M/16, in the presence of cyanide M/250

We did not observe an effect of the amine oxidase extracts on the p-hydroxyphenylisopropylamines When the *3, 4* dihydroxyphenylisopropylamine derivatives were used as substrates the only demonstrable effect was to inhibit the onset of autoxidation of the compound as compared with controls containing no extract, figure 3 The QO_2 of liver slices was not altered by these compounds

Phenol oxidase

Effect of monohydric phenyl derivatives. In these experiments the phenol oxidase activated the oxidation of only those compounds having one or two hydroxyl groups on the benzene ring Propadrine (e) which differs from

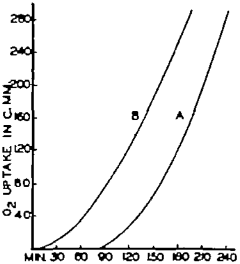


FIG 3 Illustrating the initial inhibition by liver extracts of the oxidation of cobefrin hydrochloride M/16 (A) as compared with the course of autoxidation of the compound (B) Buffered at pH 7.8, temperature = 38°C.

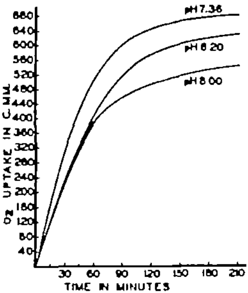


FIG 4. The effect of pH on the phenol oxidase activation of paredrine oxidation Paredrine hydrobromide M/16

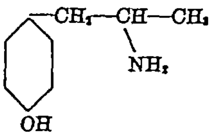
paredrine (g) in that the hydroxyl group is on the side chain is not affected by the presence of the enzyme.

Figure 4 shows the rate of reaction, O_2 uptake and effect of pH on the oxidation of paredrine (g) While a more precise attempt was not made to

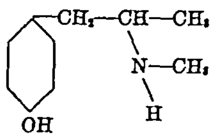
determine the optimum pH at which the enzyme catalysed this oxidation it is apparent that the optimum lies near the pH 7 36 The reaction velocity is less from the start and falls off more rapidly with the extremes of pH studied

Cyanide inhibited the catalytic action of the enzyme of both mono- and dihydroxy phenols This is in agreement with Keilin's observation (9) that 10^{-3} M HCN inhibits the action of phenol oxidase

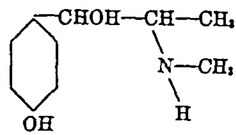
The three sympathomimetics studied which may here be considered as substituted phenols were paredrine (g), β-(4hydroxyphenyl)isopropylmethylamine (h), and suprifen (i)



(g)



(h)



(i)

If compared with the velocity of reactions catalysed by amine oxidase, the rate of reaction of this system was relatively rapid However, the character of the side chain did influence the rate of reaction among these three, as is apparent from figure 5

There is little appreciable difference in the reactions of the primary and secondary amines as may be seen However, where the side chain contains an hydroxyl group in the position beta to the amine as in (i) the initial rate of reaction is much slower than in the other two compounds While this is so, the duration of reaction is prolonged for several hours or until completion at approximately the same oxygen uptake for equimolar concentrations of the compounds (fig 5)

Though the initial oxidation is in the ring, part of the oxygen uptake is accountable for by deamination The reactivity of the amino group as well as the phenolic OH as influenced by addition of the hydroxyl group into the side chain of the molecule must then be taken into account The manner in which the polarization or electrical dissymmetry of a radical in one part of a molecule influences the molecular dipole moment of the compound is the basis for the interpretation of this observation

It is generally agreed that the "polar effect" of a radical may be exerted partly by virtue of an electric field which acts directly across space, or may also be transmitted by a process of electrostatic induction from atom to atom, or bond to bond, within the molecule It follows that a dipolar group (as the OH in (i)) could influence the reaction velocity of the parent compound (h) Since the component dipoles are vector quantities the magnitude and sign of the resultant (of the orientation, sign, and magnitude of the moments) determine the extent the reaction velocity be accelerated or

retarded. The dipole moment and sign of a secondary amino group substituted on an aliphatic chain is approximately $-1\ 00 \times 10^{-18}$ e.s.u , that for an aliphatic alcohol is $+1\ 67 \times 10^{-18}$ e.s.u and for an aromatic (OH) is +

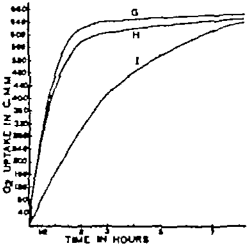


FIG 5 Illustrating the effect of the hydroxyl group on the oxidation reaction velocity of suprifen (i) as compared with those of paredrine (g) and p-hydroxyphenylisopropyl methylamine (h) Concentration of the amines = M/16, pH 7.36. Temperature = 38°C.

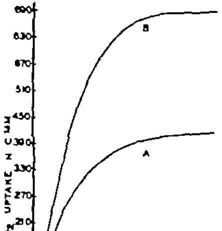
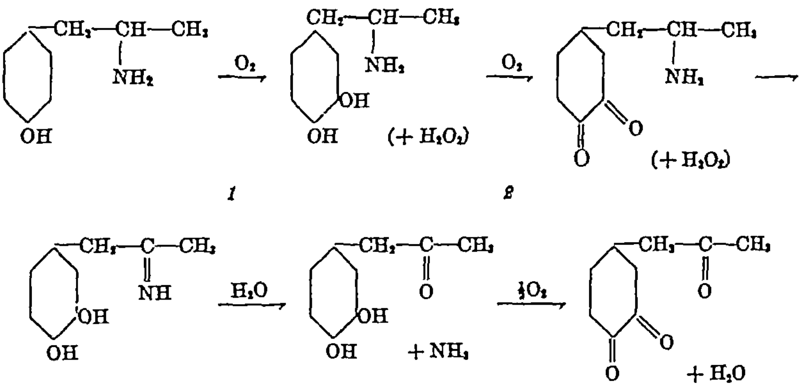


FIG 6 The course of reaction and oxygen uptake of a dihydroxyphenol cobefrin (A) as compared with a monohydroxyphenol, paredrin (B) Concentration of the amines = M/16 pH 7.36

$1\ 7 \times 10^{-18}$ e.s.u (10) The magnitude of the dipole moment induced in a chain of carbon atoms diminishes as the number of valency linkages increases. In a saturated chain the induction of polarity diminishes rapidly and is

negligible after traversing about three bonds However, the transmission of an induced polarity diminishes much less rapidly through a conjugated chain such as would probably be present between the first carbon atom of the side chain and the fourth carbon atom on the aromatic ring Is the influence of the aliphatic hydroxyl group primarily to decrease the reactivity of the aromatic hydroxyl group, inhibiting then the formation of the ortho-dihydric derivative, or does it primarily influence the deamination? We are of the opinion that while both reactions probably are involved the former is the more markedly influenced This point will be discussed below under the *ortho-dihydric phenyl derivatives*

As is illustrated in figures 4 and 6, when the oxidation of 0 2 cc of M/16 paredrine hydrobromide is completed under optimal conditions the oxygen equivalent is about 690 c mm Theoretically the O_2 uptake for 1 atom of O_2/mol of substrate = 139 c mm This would mean that 5 atom equivalents of oxygen were involved in the reaction To account for this oxygen uptake the following equation is presented



1 *2* *3* *4*

Raper (11) and Evans and Raper (12) have shown that in the oxidation of tyrosine 3,4 dihydroxyphenylalanine is produced This is analagous to step *1* It has been amply demonstrated that catechol and other ortho dihydricphenyl compounds are oxidized to ortho quinone derivatives in the course of the reaction (13), step *2* From the oxygen uptake it would appear likely that H_2O_2 was formed in the course of the reaction Pugh reported (14) that the action of tyrosinase on a monohydroxy phenolic substrate is hastened by H_2O_2 or a derivative of catechol which gives rise to ortho quinone and H_2O_2 This rôle of H_2O_2 may partially explain the fact that the purified enzymes of Keilin and Mann (4) and Kubowitz (5) oxidized monohydric

phenols either not at all or only after a latent period. If there be but a single phenol oxidase, its most marked affinity is for the ortho dihydric phenols By itself the enzyme probably has little ability to initiate the oxidation of monohydric phenols, but once the process is started reaction products such as H_2O_2 and the others mentioned catalyse the reaction Due to these agents as at least trace components of our more ample preparations we did not encounter the initial latent period.

To determine whether or not deamination occurred we used the distillation set-up described in the procedure In these experiments the tubes contained 15 cc of the phenol oxidase extract to which was added 20 cc. M/20 phosphate buffer pH 7, containing the amine The results of several experiments are given in table 1

TABLE 1

The deamination of paredrine by phenol oxidase

PAREDRINE HYDROBROMIDE	AMMONIA RECOVERED	THEORETICAL AMOUNT NH_3	PER CENT RECOVERED
mgm.	mgm.	mgm.	
50	0 96	3 68	28 3
50	1 48	3 68	40 3
75	2 18	5 49	40 0
75	2 10	5 49	38 3
100	1 07	7 40	15 0
100	1 52	7 40	20 6

While these values are low they demonstrate definitely that deamination does take place steps *3–4* *It is probable that side reactions of the ammonia* with aldehydes, for example are partially responsible for these low figures. One may conclude from these results that the ortho quinone formed on one part of the molecule of paredrine is capable of oxidising the amino group on the side chain to the corresponding imino form which in turn reacts with water, liberating ammonia and replacing the nitrogen with an atom of oxygen to form the corresponding ketone The ortho dihydric group is simultaneously oxidized again to the ortho quinone state (step *5*)

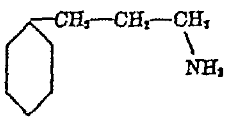
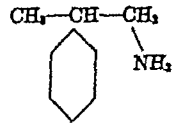
It is probable that once the quinone is formed only one atom of oxygen per mol is necessary to maintain it in the oxidation-reduction system resulting in deamination of the side chain Evidence for this is the finding that if one starts with a *3 4* dihydroxy compound (cobefrin) analagous to the second step of the equation, 3 atoms of oxygen are used per mol of substrate Figure 6 shows the oxidation of cobefrin completed at an oxygen uptake of about 425 c.mm. Theoretically the equivalent O_2 uptake for 1 atom of O_2/mol of substrate = 137 c.mm. The atoms of oxygen per molecule of cobefrin

from the calculations are 3 This equation is not necessarily meant to include all possible steps in the reaction or the final products

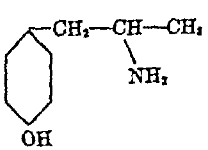
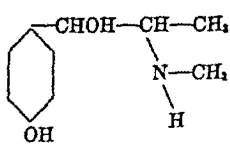
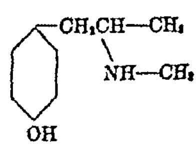
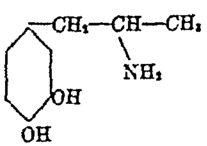
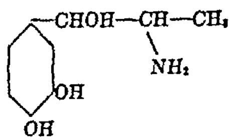
Ortho dihydric phenyl derivatives The oxidation of the two *3,4* dihydroxyphenylisopropylamines in the presence of phenol oxidase is similar to that for paredrine Figure 6 compares the rate of reaction and O_2 uptake for paredrine and cobefrin equimolar It may be noted that the reaction velocity of the two is similar The O_2 uptake in atoms per mol of cobefrin is 3, indicating that it fits in with the oxidation scheme set forth in the equations The reaction velocity for *3,4* dihydroxyphenylisopropylamine is only very slightly more rapid than for the corresponding *3,4* dihydroxyphenylisopropanolamine The two curves are almost superimposable, consequently the one has been omitted This would indicate that the introduction of the aliphatic hydroxyl group does not materially influence the reactivity of the amino group Also, the aliphatic hydroxyl radical does not influence the oxidation of the ortho dihydric derivative However, in figure 5, the reaction velocity of (h) *4*-hydroxyphenylisopropylmethylamine is much greater than that of (i) *4*-hydroxyphenylisopropanolmethylamine. One must conclude, then, that the influence of introducing an aliphatic hydroxyl group into these compounds is primarily to retard the initial oxidation of the monohydric derivatives to the corresponding ortho dihydroxyphenylisopropylamines

DISCUSSION

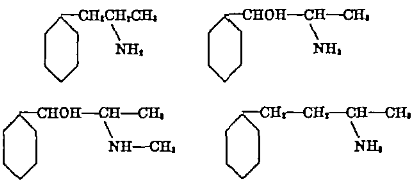
A classification of the amines studied according to the results presented here would cause them to fall into three groups A Those which were oxidised in the presence of amine oxidase

B Those whose oxidation was activated by phenol oxidase

C Those not oxidised directly by either enzyme system



It proves interesting to examine certain literature concerning some of these agents in the light of the findings reported here. In 1929 Chen, Wu and Hendriksen (18) reported from an extensive study that the presence of a methyl group on the alpha carbon atom of β-phenylethylamine confers on that compound the ability to produce systemic effects in man when given orally as evidenced by ephedrine and nor-ephedrine The following year basing their conclusions on clinical observations primarily, Piness, Miller and Alles (19) similarly reported that the addition of a methyl group on the side chain of β-phenylethylamine was responsible for the duration of action and effectiveness of ephedrine and amphetamine when administered orally These compounds fall into the third group C These we found were not oxidised in the presence of either amine oxidase or phenol oxidase

We have recently reported that benzedrine (amphetamine) is excreted as such by the kidneys (20) Also Richter reported the excretion of ephedrine following its administration (21) Again these two compounds belong to the group not inactivated by either system.

Blaschko, Richter and Schlossman (8) have demonstrated the distribution of amine oxidase in mammalian tissues including the gastrointestinal tract, and Bhagvat, Blaschko and Richter (22) have extended further the known distribution of the enzyme. Block (23) Pugh (24), Charles (25) and Charles and Rowles (26) have presented experimental evidence in support of the functional rôle of phenol oxidase in animals though recently Bhagvat and Richter (24) have questioned its significance

There seems little doubt, then, that these and probably other such systems are responsible for the determination of whether a sympathomimetic amine should be given orally or injected to be effective physiologically Likewise the fact that certain phenylisopropylamines are excreted as such may be attributed in part to their refractoriness to enzymes which would completely inactivate closely related amines.

SUMMARY

It has been shown that the inactivation of different sympathomimetic compounds by the action of amine oxidase or phenol oxidase is dependent on their molecular configuration

Those phenylpropylamines having the amino group on the terminal carbon atom of the side chain were oxidised in the presence of amine oxidase and oxygen

The compounds having one or two hydroxyl groups on the benzene ring were oxidised in the presence of phenol oxidase

If the amino group was on the carbon atom adjacent to the terminal one, and the molecule contained no hydroxyl groups on the ring the compound was not inactivated by either system

Evidence has been presented to support the concept that amine oxidase and phenol oxidase or similar systems determine the oral efficacy and the excretion of these chemicals, that is in general, the action and fate of these sympathomimetic amines in the body

ACKNOWLEDGMENT

The author wishes to express his appreciation for the gifts of amines so generously supplied by the following individuals and concerns Dr Gordon A Alles (c), (d), (h) and (j), Dr T B Wallace of Smith, Kline and French, Inc (b), (c), (d), and (g), Dr Wm A Feirer, Sharp and Dohme (a), (c) and (e), and to Dr O W Barlow, Winthrop Chemical Co (i) and (k)

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THE ENZYMATIC DEACETYLATION OF HEROIN AND RELATED MORPHINE DERIVATIVES BY BLOOD SERUM[1]

C I WRIGHT

Pharmacologist, Division of Pharmacology, National Institute of Health, U S Public Health Service, Washington, D C

Received for publication November 19, 1940

About five years ago, Eddy and Howes (1) and Wright and Barbour (2) suggested the possibility that diacetylmorphine might be partially deacetylated after injection into the body This suggestion, based on the ease with which the phenolic acetyl[2] is removed chemically, was one explanation offered for the equal potency of monoacetylmorphine and diacetylmorphine as found in a study of the relationship between chemical structure and physiological activity of the acetylated morphine derivatives In view of this assumption it was of considerable interest to find, while investigating the effect of morphine and heroin on the activity of cholinesterase, that blood serum contains an enzyme capable of causing a rapid removal of the acetyl groups of heroin Consequently, the work was extended to include measurements of the rate of hydrolysis of diacetyldihydromorphine, monoacetylmorphine and monoacetyldihydromorphine It was found that sera from different rabbits differ in their deacetylating ability when heroin, diacetyldihydromorphine and monoacetylmorphine were used as substrates, but no serum was found capable of deacetylating monoacetyldihydromorphine

METHODS

The measurements of the rates of deacetylation were made with Barcroft differential manometers in essentially the same manner that Ammon (3) measured choline esterase activity The method depends on a reaction of the liberated acetic acid with the bicarbonate of the solution resulting in an evolution of carbon dioxide which is measured manometrically The manometer flasks, of approximately 17 cc capacity, had side arms which permitted the separation of the substrates from the serum until temperature and gaseous equilibria were attained The salts of the morphine derivatives formed weakly acid solutions and therefore were usually placed in the main compartments of the flask, dissolved in the bicarbonate Ringer's solution, so that the liberation of carbon dioxide due to simple mixing might occur before the experimental period began The total fluid volume was always 3 0 cc but the quantity of solution in the main vessel and

[1] A preliminary report of this work appeared in Science, *92*, *244*, *1940*

[2] For convenience, the acetyl radicals, replacing the hydrogen of the phenolic hydroxyl and alcoholic hydroxyl of morphine, will be called *phenolic acetyl* and *alcoholic acetyl* respectively

side arms was varied to suit the purposes of the experiment. Determinations were made at 37.5°C and the bottles were thoroughly flushed with a water-saturated mixture of 95 per cent nitrogen (or oxygen) and 5 per cent carbon dioxide.

Blood (8 to 10 cc.) was collected from the marginal ear vein of the rabbit, allowed to clot and then placed in the refrigerator over night. The following morning the serum was taken off and unless perfectly clear was centrifuged to throw out the red cells. The blood from the human subjects was collected from the median basilic vein of the forearm and the serum obtained as from the rabbit blood.

The rabbits were all albinos of apparently the same breed and were kept under the same conditions. They were fed pellets of Purina rabbit chow and cabbage and were allowed free access to water.

Chemical data

The acetylated morphine derivatives used in this investigation as well as the following chemical descriptions of them were furnished by Doctor L. F. Small of the National Institute of Health.

Heroin. No. 1. Diacetylmorphine was prepared by acetylation of pure anhydrous morphine with acetic anhydride in boiling benzene, and recrystallized from acetone, m.p. 169 to 170. It was converted to the hydrochloride in acetone by cautious addition of alcoholic hydrogen chloride to positive Congo red reaction (Schwyzer, 'Die Fabrikation der Alkaloide,' pp. 39-48, Springer, Berlin, 1927). The salt melted at 227 to 229 and showed $(\alpha)_D^{25}$ -147.5 in aqueous solution ($c = 1.539$).

No. 2. Anhydrous morphine prepared from a highly purified sample of morphine sulfate having $(\alpha)_D^{25}$ -98.5 was acetylated with acetic anhydride in pyridine at room temperature (24 hours). Acetic anhydride and pyridine were removed at 30 in a vacuum, and the diacetylmorphine was precipitated with sodium bicarbonate solution and purified from ethyl acetate m.p. 170 to 171, $(\alpha)_D^{25}$ -166.4 (methanol, $c = 1.490$). The hydrochloride was prepared in acetone solution with alcoholic hydrogen chloride added in such amount that the solution never became acid to litmus. Base which was not converted to salt remained in solution in the acetone, or was removed in the subsequent washing with absolute ether. Analysis showed the salt to be anhydrous. It melted at 230 to 232° $(\alpha)_D^{25}$ -150 (water, $c = 1.004$).

No. 3. On the assumption that local excess of acid in the preparation of no. 2 might have caused partial hydrolysis, a third procedure was used. Highly purified morphine (see no. 2) was converted to the hydrochloride, which was recrystallized twice and dehydrated at 100 in a high vacuum. The hydrochloride was acetylated in pyridine with acetic anhydride (48 hours, room temperature), the solvent removed in a vacuum at 30, and the heroin precipitated by slow addition of absolute ether. The product was dissolved in absolute alcohol at 30 and was twice precipitated with absolute ether. It was anhydrous, m.p. 243 to 244, $(\alpha)_D^{24}$ -156.3 (water, $c = 1.044$).

It will be noted that with increasingly gentle preparative procedures, both melting point and specific rotations of the products are progressively raised.

Dihydroheroin. Anhydrous dihydromorphine was acetylated with acetic anhydride in boiling benzene (3 hours). The resulting diacetyldihydromorphine was converted to the hydrochloride with alcoholic hydrogen chloride in acetone. The salt melted at 217° and had $(\alpha)^{25}$ -59.3 (water, $c = 1.203$).

Monoacetylmorphine hydrochloride. Diacetylmorphine in alcohol solution, was treated with two equivalents of a concentrated aqueous solution of hydroxylamine hydrochloride. The hydrochloride of monoacetylmorphine crystallized out after short standing. It was purified by three crystallizations from water; the salt contained 12.39 per cent water (about $2\frac{1}{2}$ H_2O), m.p. 293°, specific rotation (anhydrous) $(\alpha)_D^{25}$ -153 (water, $c = 0.7$).

Monoacetyldihydromorphine hydrochloride Prepared from diacetyldihydromorphine with hydroxylamine hydrochloride as described above and purified from 40 per cent alcohol, $(\alpha)_D^{*}$ − 111 7° (water, c = 1 067)

The data on the deacetylation of heroin in this report, with the exception of tables 1 and 2, were all obtained with heroin sample no 1 The other two samples of heroin were prepared in an attempt to explain the low yield of carbon dioxide discussed in the next section of the report

RESULTS

Figure 1 is a graphic presentation of one of the preliminary experiments carried out to test the effect of heroin on the activity of cholinesterase When

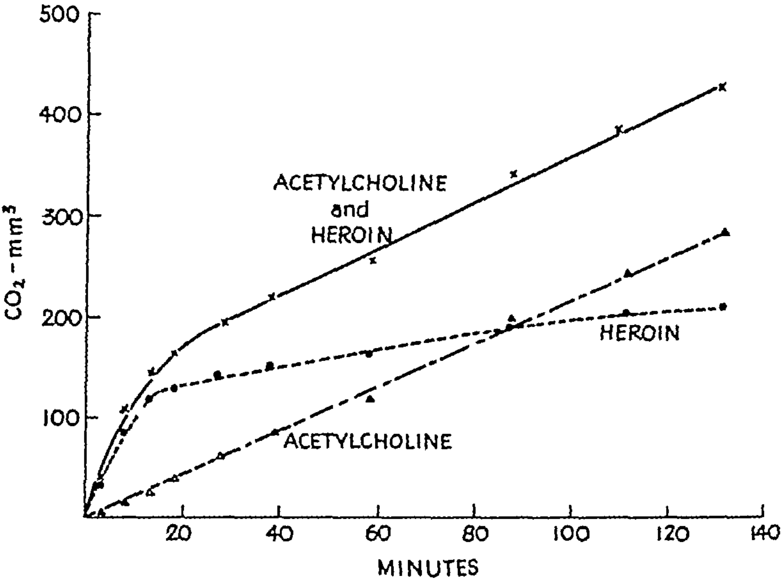

FIG 1 THE RATE OF HYDROLYSIS OF HEROIN AND ACETYLCHOLINE, SEPARATELY AND IN COMBINATION, BY RABBIT SERUM

The concentrations used were Acetylcholine 11 0 mgm , heroin 2 5 mgm and serum 0 25 cc in a total volume of 3 0 cc

rabbit serum was added to acetylcholine alone carbon dioxide was liberated at a uniform rate for a period of two hours When 2 5 mgm of heroin was included with the acetylcholine, the rate of liberation of carbon dioxide was very rapid during the first 20 minutes and then closely paralleled the rate for acetylcholine alone *Addition of serum* to heroin without acetylcholine showed that the initial rapid release of carbon dioxide was due to the deacetylation of the heroin The fact that the upper and lower curves run almost parallel after 30 minutes indicates that an exposure to heroin has little if any inhibiting action on serum cholinesterase under the conditions of the experiment

Figure 2 shows the rate of deacetylation of heroin by sera from six rabbits, identified by numbers on this and all the graphs excepting Figure 7 The rabbit sera fall into two groups based on their deacetylating ability Sera from three of the animals (297, 294 and 277) deacetylated heroin more rapidly and more completely than sera from the other three (275, 276 and 295) Closer inspection of the curves shows that the amount of CO_2 liberated by the first group was almost exactly double that liberated by the second group There is also a rather sharp break in the upper three curves indicating two separate rates of deacetylation. It was natural then to assume that three

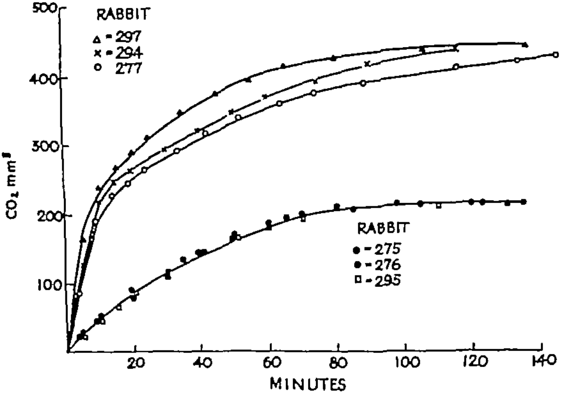

FIG 2. THE DIFFERENCE IN THE RATE AND EXTENT OF HYDROLYSIS OF DIACETYLMORPHINE BY SERA FROM SIX RABBITS

Each determination was made with 0.5 cc of serum acting on 5 0 mgm of diacetyl morphine (heroin) at 37.5°C The identification numbers of the rabbits are given on the graph.

of the rabbit sera (275 276 and 295) were able to remove but one acetyl group from diacetylmorphine while the other three were able to split off both acetyls If the sera that were able to remove but one acetyl were acting only on the phenolic acetyl group (carbon-3) they should not be able to hydrolyze monoacetylmorphine. The sera from the other group of animals should however readily attack monoacetylmorphine and remove the alcoholic acetyl radical (carbon-6) Consequently a series of determinations were made using monoacetylmorphine as substrate instead of diacetylmorphine.

Figure 3 shows that rabbits 297 294 and 277 were able to deacetylate monoacetylmorphine. The upper two curves represent the rate of hydrolysis

using 5 0 mgm of monoacetylmorphine and 0 5 cc of serum The lower curve was obtained with 2 5 mgm of monoacetylmorphine and 0 5 cc of serum Under the same conditions the sera from animals numbered 275, 276 and 295 were unable to deacetylate monoacetylmorphine It seems clear then that those animals able to remove but one acetyl from heroin split off only the more labile phenolic acetyl group while the other three animals are able to form acetic acid by hydrolysis at both the phenolic and alcoholic positions of the molecule

Figure 4 (A and B) shows the rate of liberation of CO_2 from 5 0 mgm of diacetylmorphine in the presence of increasing concentrations of serum and

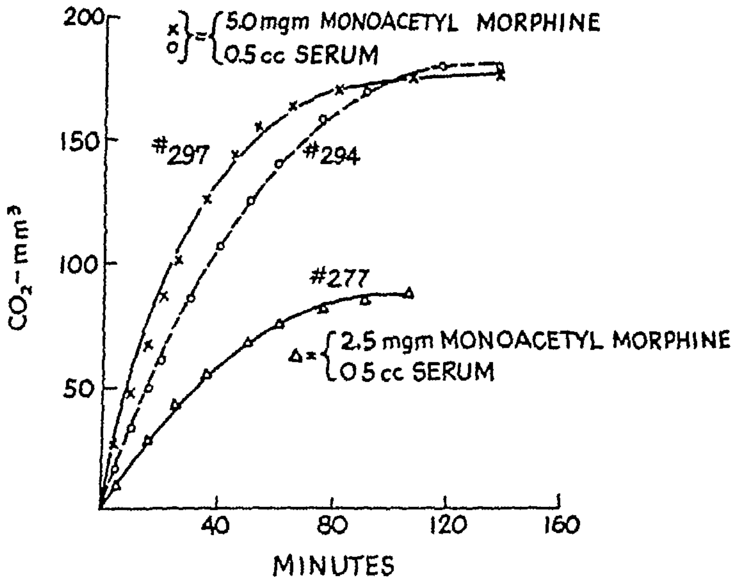

FIG 3 THE DEACETYLATION OF MONOACETYLMORPHINE BY RABBITS 297, 294 AND 277 Animals numbered 295, 275 and 276 (fig 1) did not hydrolyze monoacetylmorphine

(C and D) the rate and quantity of CO_2 liberated when increasing quantities of heroin are added to 0 5 cc of serum Comparable determinations have been made using sera from the other four animals with similar results The rate of deacetylation increases rapidly with increasing serum concentration whether the hydrolysis is taking place at the 3-carbon (A) or at both the 3 and 6 carbons (B) The quantity of CO_2 liberated, when hydrolysis had ceased, is proportional to the amount of heroin added (C and D) and equal to 75 to 80 per cent of the theoretical amount for one acetyl group in the case of rabbit 276 and to 75 to 80 per cent of the theoretical for liberation of both acetyls when serum from rabbit 297 is used

Diacetyldihydromorphine differs from heroin in the loss of the double bond in the 7–8 position through hydrogenation. When this compound is used as a substrate, deacetylation takes place as shown in figure 5 It is obvious that the rabbit sera fall again into two groups on the basis of the rate of hydrolysis but, unlike the results obtained with heroin (fig 2), the amount

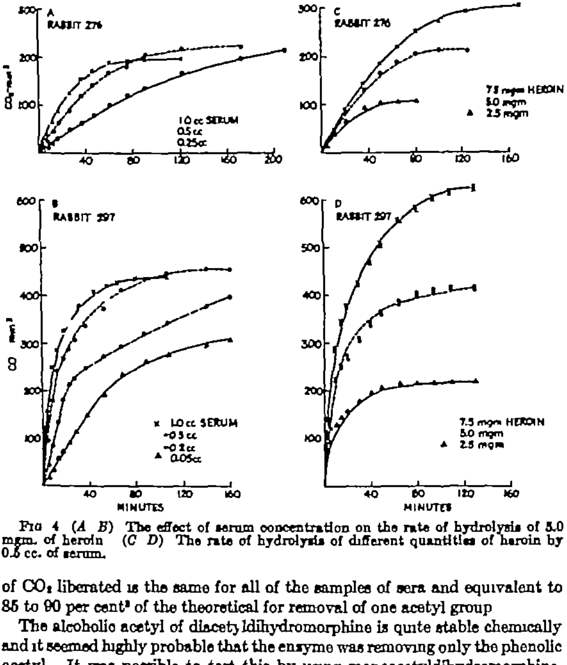

FIG 4 (*A* *B*) The effect of serum concentration on the rate of hydrolysis of 5.0 mgm. of heroin (*C* *D*) The rate of hydrolysis of different quantities of heroin by 0.5 cc. of serum.

of CO_2 liberated is the same for all of the samples of sera and equivalent to 85 to 90 per cent[3] of the theoretical for removal of one acetyl group

The alcoholic acetyl of diacetyldihydromorphine is quite stable chemically and it seemed highly probable that the enzyme was removing only the phenolic acetyl. It was possible to test this by using monoacetyldihydromorphine which has only an alcoholic acetyl radical, as substrate. This compound was

[3] The higher value given in the preliminary report of this work (Science 92 1940) was calculated from an incorrect value for the molecular weight of the compound.

not hydrolyzed by sera from any of the rabbits, proving that dihydrodiacetylmorphine was deacetylated only at the phenolic position

In order to investigate more carefully the low yield of carbon dioxide from the acetylated morphine derivatives, a series of determinations was made, after recalibration of the apparatus and minor improvements in the technique, designed to give more precise measurements of the total carbon dioxide evolved For this work Doctor L F Small kindly prepared samples 2 and 3 of heroin as described above

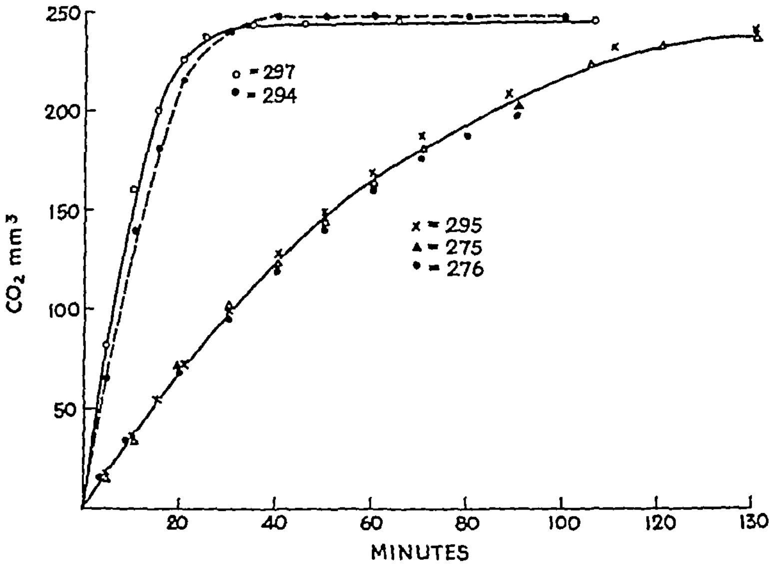

FIG 5 THE RATE OF REMOVAL OF ONE ACETYL GROUP FROM DIACETYLDIHYDROMORPHINE

The identification numbers of the rabbits are indicated below the curves 5 0 mgm of diacetyldihydromorphine and 0 5 cc of rabbit serum were used for each determination

The manometers were calibrated by tipping an excess of H_2SO_4 (0 5 cc of 0 07 N) from the side arm into the main compartment of the manometer flask, containing an accurately made and measured sodium bicarbonate solution A check of the calibration (table 1) was made preliminary to the measurements listed in table 2 and the lower half of table 1 From the check determinations it is evident that the maximum variation of a single value from the average is within one per cent and the variation of the average from the theoretical is negligible

A portion of the discrepancy between the theoretical amount of CO and the actual values can be accounted for by the retention of CO_2 by serum (4, 5)

In order to measure this retention value, determinations were made of the liberation of CO_2 by a known quantity of acetic acid when added to an excess of sodium bicarbonate, as bicarbonate Ringer's solution (6) with and without added serum The results are given in the lower half of table 1 The first series of values obtained by the addition of 0 0345 N acetic acid to 2.5 cc. of Ringer's solution, shows that only 97 per cent of the theoretical amount of carbon dioxide is liberated The addition of 0 4 0.5 or 0 6 cc. of the same acetic acid to Ringer's solution plus 0 5 cc of rabbit serum liberated only 87 5 per cent of the theoretical amount of carbon dioxide Thus, 3 per cent of the missing carbon dioxide can probably be attributed to incomplete

TABLE 1

Carbon dioxide formed from sodium bicarbonate by sulfuric acid

TRIALS	H_2SO_4 (0.07 N)	$NaHCO_3$	CO_2 Average	CO_2 Range	CO_2 Theoretical
	cc.	mgm.	mm.	mm.	mm.
12	0 5	1 25	332	(330–334)	333
6	0 5	1 375	366	(365–360)	367

Carbon dioxide formed from bicarbonate Ringer by acetic acid (with and without serum)

TRIALS	RABBIT	CH_3COOH	RINGERS	SERUM	CO_2 Average	CO_2 Range	CO_2 Theoretical	CO_2 Per cent theoretical
		cc.	cc.	cc.	mm.	mm.	mm.	
9		0 50	2 5	None	375	(372–378)	387	97 0
3	295	0 50	2 0	0 5	338	(335–341)	387	87 5
4	277 295	0 60	1 9	0 5	404	(398–410)	464	87 0
2	277	0 40	2 1	0 5	271	(268–274)	310	87 5

Titrated with 0 100 N NaOH (phenolphthalein) the acetic acid was 0 0345 normal

neutralization of the acetic acid under the conditions of the experiments and an additional 10 per cent is retained due to the presence of the serum.

Based on the results presented in table 1 the yield of carbon dioxide from the deacetylation of the acetylated morphine derivatives should be very nearly 87 per cent of the theoretical amount. The results of actual determination of the CO_2 evolved from heroin and diacetyldihydromorphine in the presence of 0 5 cc. of serum are given in table 2 The first four trials, using serum from a rabbit (295) that deacetylated only at the 3-carbon yielded on the average 76 per cent of the theoretical value. In the next group of nine determinations, using heroin sample 2 78 per cent of the theoretical value was obtained In another group of 10 determinations using heroin sample 3 80.5 per cent of the theoretical amount of CO_2 was obtained

Under the same experimental conditions diacetyldihydromorphine liberated 87 per cent of the theoretical quantity of CO_2

Since, as shown in table 1, acetic acid itself yields only 87 5 per cent of the theoretical amount of CO_2 in the presence of 0 5 cc of serum, the liberation of acetic acid from diacetyldihydromorphine must have been complete The yield of acetic acid from heroin on the other hand falls short of the expected value by 7 to 11 per cent depending on the sample of heroin used and the activity of the serum Since 11 per cent is missing when a rabbit is used

TABLE 2

Carbon dioxide formed from sodium bicarbonate by enzymatic deacetylation of heroin and diacetyldihydromorphine

TRIALS	RABBIT	HEROIN	SERUM	CO_2 Average	CO_2 Range	THEORETICAL	PER CENT THEORETICAL	REMARKS
Heroin*								
		mgm	cc	mm^3	mm^3	mm^3		
4	295	5 0 (2)	0 5	210	(208–212)	276	76 2	Monoacetyl rabbit‡
9	297 277 294	5 0 (2)	0 5	430	(423–437)	552	78 0	Diacetyl rabbits§
10	294 297 277	5 0 (3)	0 5	444	(440–454)	552	80 5	Diacetyl rabbits§
Diacetyldihydromorphine†								
8	297	5 0	0 5	239	(231–247)	275	87 0	

* Anhydrous diacetylmorphine, molecular weight = 405 64

† Anhydrous diacetyldihydromorphine, molecular weight = 407 66

‡ Serum from this animal removes but one acetyl radical

§ Sera from these animals remove both acetyl radicals

Figures in parenthesis indicate sample number of heroin

that deacetylates only at the 3-carbon and 7 to 9 per cent when the serum deacetylates at both the 3 and 6 carbons there is a possibility that a slight autohydrolysis takes place at the 3-carbon previous to the experimental determination of the CO_2

Figure 8 shows the rate of deacetylation of heroin and acetylcholine by sera from two human subjects While heroin is deacetylated at a much slower rate than by rabbit sera, acetylcholine is hydrolyzed at a greater rate as can be seen by comparison with figure 8 and from the results published by Hall and Lucas (7) Several other samples of human sera were tested and found

to have practically the same deacetylating ability as the examples given in figure 6

Physostigmine has a marked inhibiting action on the enzyme responsible for the hydrolysis of the acetylated morphine derivatives In the experiment shown (fig 7) the same concentrations of sera and substrate were used as in figures 2, 3 and 5 but in addition 0 1 mgm. of physostigmine was added to the total volume of 3 0 cc. Detectable inhibition can be obtained when as little as 0.001 mgm. of physostigmine is added previous to mixing the serum and substrate.

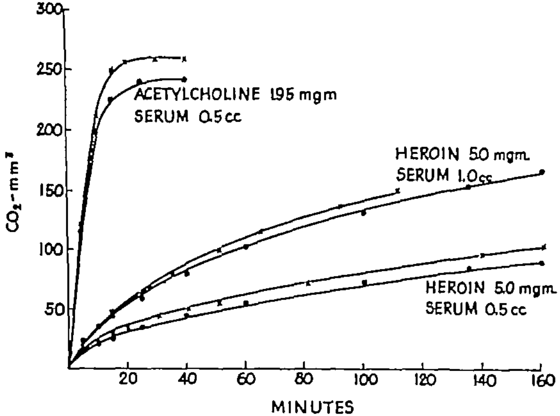

FIG 6 THE RATE OF DEACETYLATION OF HEROIN AND ACETYLCHOLINE BY NORMAL HUMAN SERUM FROM TWO SUBJECTS

The enzyme is heat sensitive Subjecting the serum to 60°C for 5 minutes decreases its enzymatic activity and heating to that temperature for 30 minutes almost completely destroys its hydrolyzing ability

While sera from the rabbits investigated fall into two groups as regards their ability to hydrolyze the acetylated morphine compounds, there appears to be no such distinct difference in their ability to hydrolyze acetylcholine (fig. 8) As shown by the graph, when acetylcholine is used as substrate, the rate of CO_2 production is very nearly the same on addition of serum from any one of the animals. Repetition of the determinations has shown that the concentration of cholinesterase varied but little over a period of weeks

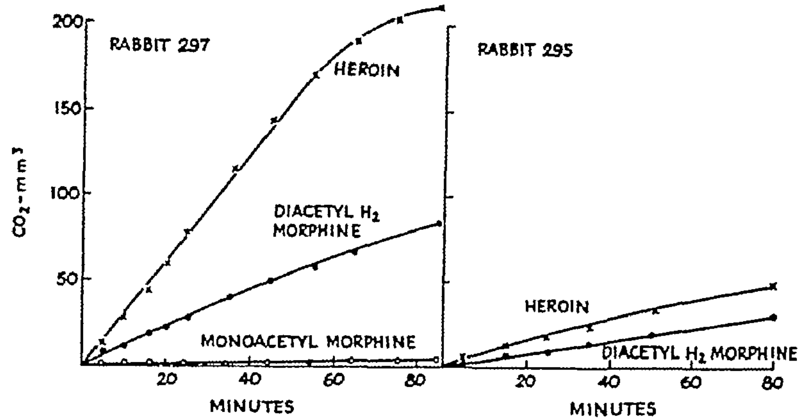

FIG 7 THE INHIBITORY ACTION OF PHYSOSTIGMINE ON THE ESTERASE THAT HYDROLYZES THE ACETYLMORPHINES

In each determination 0 5 cc of serum and 0 10 mgm of physostigmine were added to a total volume of 3 0 cc 5 0 mgm of the acetylated morphine indicated for each curve was added as a substrate Compare with rabbits numbered 297 and 295 of figures 2, 3 and 5

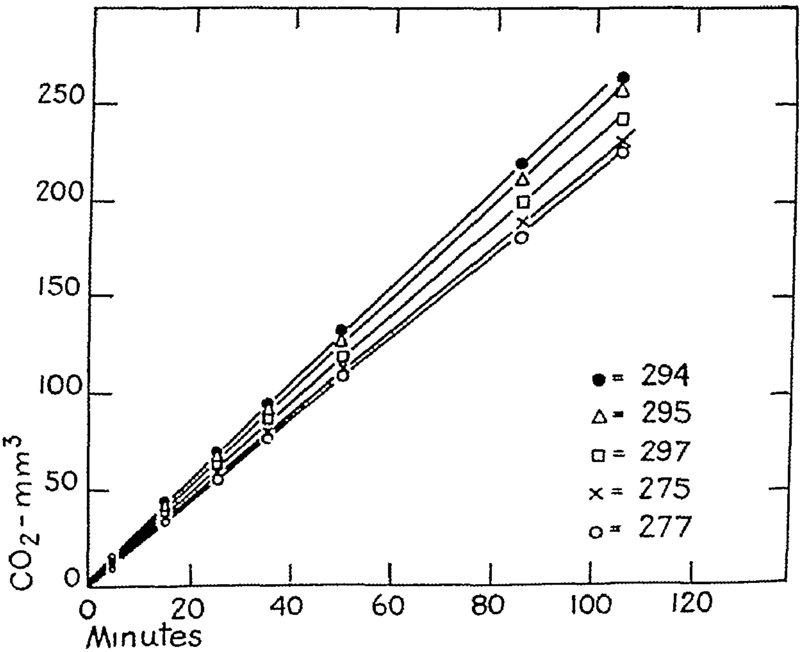

FIG 8 THE RATE OF HYDROLYSIS OF ACETYLCHOLINE BY SERA FROM RABBITS 294, 295, 297, 275 AND 277

Each determination was made with 0 25 cc of serum and 12 5 mgm of acetylcholine hydrochloride in a total volume of 3 0 cc

and the order of increasing concentration, for such slight differences as were found, was not the same order as given in figures 2 or 5 when heroin and diacetyldihydromorphine were used as substrates.

DISCUSSION

The ability of serum to deacetylate some of the acetylated morphine derivatives opens up a wide field for speculation and further work on possible changes that other morphine derivatives might undergo when introduced into the body It is obvious from the results presented that diacetylmorphine is in all probability converted to morphine on entering the blood stream of some rabbits and on the other hand, only to monoacetylmorphine in others. Then again, as preliminary experiments on the concentration of the enzyme in the tissues have already indicated the tissues of the animal may contain the enzyme essential for removal of the alcoholic acetyl group in which case monoacetylmorphine would also be converted to morphine. It is a possibility, at least, that the reduced morphines (desoxymorphines) or the oxidized morphines (dilaudid) are converted into morphine in the body by means of oxidizing or reducing enzyme systems Many of the differences in physiological activity noted among the morphine derivatives would then have to be explained on a physical basis rather than on chemical structure exclusively

It is well known (1 2 and references) that, by the usual methods of administration, heroin is a much more active drug than morphine as judged by the minimal effective dose necessary to elicit a given effect. It is also common knowledge that heroin has a high addiction liability If it is assumed that heroin is converted to morphine on entering the blood stream, as is suggested by the evidence presented here then these differences must be due to differences in the physical properties of the material originally injected. It was found in preliminary work that morphine salts were rapidly changed to the base and precipitated in solutions containing bicarbonate at the concentration found in Ringer's solution Heroin on the other hand was more soluble. The explanation of the difference in potency might rest on this factor at least as regards subcutaneous injections. The morphine is possibly precipitated in the presence of the bicarbonate of the tissues and feeds into the blood stream slowly while the more soluble heroin passes into the blood readily and after hydrolysis, is present as morphine in a relatively high concentration. Such an explanation is in line with the greater speed of action and short duration of action found for heroin and similar compounds, and suggested (8) as an explanation for their high addiction liability Comparison of the respiratory depressant and analgesic effectiveness of morphine and heroin injected intravenously in the minimal effective dose range would be interesting in this respect. Guinard (9) and Mayor (10) found the lethal dose of morphine for the rabbit to be 5 to 10 times as great as that for heroin when injected intravenously Mayor injected into the lateral ear vein at a rate of 12 mgm. per minute. Under such conditions there would be the possibility of a pre-

cipitation of the morphine and an incomplete deacetylation of the heroin By intracerebral injection of much smaller quantities (0 1 to 3 5 mgm) Mayor's results (11) indicate that morphine is more toxic than heroin

Ammon (12) has stated that inhibition by physostigmine in low concentration is a specific test for cholinesterase By this criterion the enzyme responsible for hydrolysis of the acetylated morphine derivatives is cholinesterase However, rabbit sera that have almost identical ability to hydrolyze acetylcholine (fig 8) differ from one another qualitatively in their capacity to deacetylate heroin (fig 2) and monoacetylmorphine (fig 3) and quantitatively in ability to hydrolyze diacetyldihydromorphine (fig 5) The qualitative difference indicates the possible presence of two enzymes one of which may or may not be cholinesterase The fact that only three of the sera, out of six containing cholinesterase, are able to deacetylate monoacetylmorphine (fig 3) indicates that cholinesterase is not the enzyme responsible for removing the alcoholic acetyl radical On the other hand, the widely unequal rates of deacetylation of the phenolic acetyl when heroin or diacetyldihydromorphine are used as substrates indicates that cholinesterase is not the enzyme that removes the phenolic acetyl From the data at hand it might be *tentatively* concluded that two enzymes are involved in the hydrolysis of the acetyl morphine derivatives neither of which is cholinesterase It is admitted that other explanations are possible and more definite conclusions must await further work on the activity of the enzymes and comparisons with other esterases of the blood

The explanation (1, 2) of the pharmacological similarity between diacetyl morphine and monoacetylmorphine, based on the assumption that the diacetylmorphine is converted to monoacetylmorphine in the body, is open to question in view of the work reported here It is more likely that the similarity is due to the fact that both substances are converted to morphine by deacetylating enzymes and both reach the sensitive tissues at the same rate and concentration

The unexplained decrease in physiological activity (1, 2) brought about through hydrogenation of diacetylmorphine and monoacetylmorphine might be explained by the fact that the hydrogenated compounds are not completely deacetylated by blood sera (or tissues so far investigated) Therefore these compounds are not readily converted to morphine in the body and act as dihydromonoacetylmorphine or are too slowly hydrolyzed to afford a high concentration of morphine

SUMMARY

The blood sera of rabbits contain an enzyme or enzymes which catalyze the deacetylation of some acetylated derivatives of morphine The sera are readily divisible into two classes based on their deacetylating ability One class removed both acetyl radicals from diacetylmorphine, deacetylated

monoacetylmorphine and removed the phenolic acetyl from diacetyldihydromorphine. The other class removed only the phenolic acetyl from diacetyl morphine, did not deacetylate monoacetylmorphine and only slowly removed the phenolic acetyl from diacetyldihydromorphine None of the sera was able to remove the alcoholic acetyl radical from monoacetyldihydromorphine

The bearing of the presence of the deacetylating enzymes on the problem of the varying potency of the morphine derivatives and the question of relationship to cholinesterase is discussed

It is a pleasure to acknowledge my indebtedness to Doctor L F Small of the National Institute of Health for furnishing the acetylated morphine derivatives and for helpful consultations on the chemistry of the compounds.

This work is a continuation of the studies on the pharmacology of morphine derivatives, sponsored during the past ten years by the Committee on Drug Addiction of the National Research Council.

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THE ACTION OF BERBERINE ON MAMMALIAN HEARTS

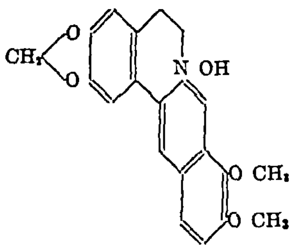
CHANG-SHAW JANG

From the Department of Pharmacology, Oxford

Received for publication July 31, 1940

In previous studies (1) it was found that berberine following doses of ergotoxine causes a rise instead of a fall of blood pressure in dogs, in other words, the usual depressor effect of berberine can be reversed by ergotoxine This reversal is independent of the central nervous system, and other findings suggest that the heart may play an important rôle in it In the course of further study of this phenomenon some incidental observations on the cardiac action of berberine and its interaction with acetylcholine seemed more important and interesting than the original problem, and this led to the present study

Berberine is a yellow and bitter alkaloid, widely distributed in nature, particularly in the various species of berberis plants (such as Berberis aristata and B vulgaris) and Hydrastis canadensis ("Golden seal") In the latter it occurs to the extent of nearly 2 5 per cent Berberine base and some of its salts are fairly soluble in water, but a strong solution of berberine sulphate forms a heavy precipitate when it comes into contact with normal saline or blood In our experiments we used a 1 per cent solution of berberine sulphate in distilled water It is chemically related to papaverine and hydrastine, and its structural formula is probably as follows



Early literature on its pharmacological properties was reviewed in Heffter's Handbuch (2) Since the establishment of its chemotherapeutic value in cutaneous leishmaniasis (3, 4, 5, 6), the pharmacological interest in this drug has been renewed and a number of papers have been published in recent years (7, 8, 9, 10, 11, 12, 13)

EXPERIMENTAL RESULTS

1 *The spinal cat*

Confirming our previous results in dogs, it was found that moderate doses of berberine caused a fall in blood pressure accompanied by an increase of

splenic volume and an augmentation of the splenic rhythmic contractions. The reversal of this depressor effect of berberine after ergotoxine was, however, not so constant as in anaesthetised dogs, and the berberine effect on the splenic volume was merely abolished but not definitely reversed (fig 1)

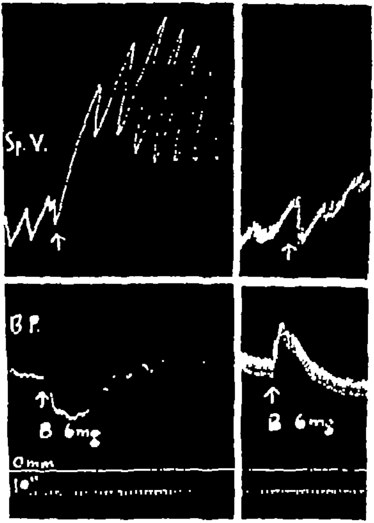


FIG 1 SPINAL CAT
Upper tracings, spleen volume. Lower blood pressure Effects of equal doses of berberine (*B*) before and after 3 mgm ergotoxine ethanesulphonate in divided doses.

2 Heart lung preparation

Starling's heart lung preparation was made from the dog. The left auricular pressure was recorded by a saline manometer and a piston recorder In some experiments a Morawitz cannula was introduced into the coronary sinus to measure the coronary flow and in others the volume of the heart was measured by a Henderson cardiometer The arterial resistance was adjusted to produce a constant arterial pressure of 80 to 100 mm Hg. The amount of venous inflow was adjusted to the size of the heart and it varied from 300 to

500 cc per minute The total amount of circulating blood was from 800 to 1000 cc Drugs were injected into the cannula in the superior vena cava

In small and moderate doses (1 to 10 mgm) berberine always stimulated the heart and its stimulant effect was comparable in intensity to that produced by 1 to 2 micrograms of adrenaline but much more prolonged It

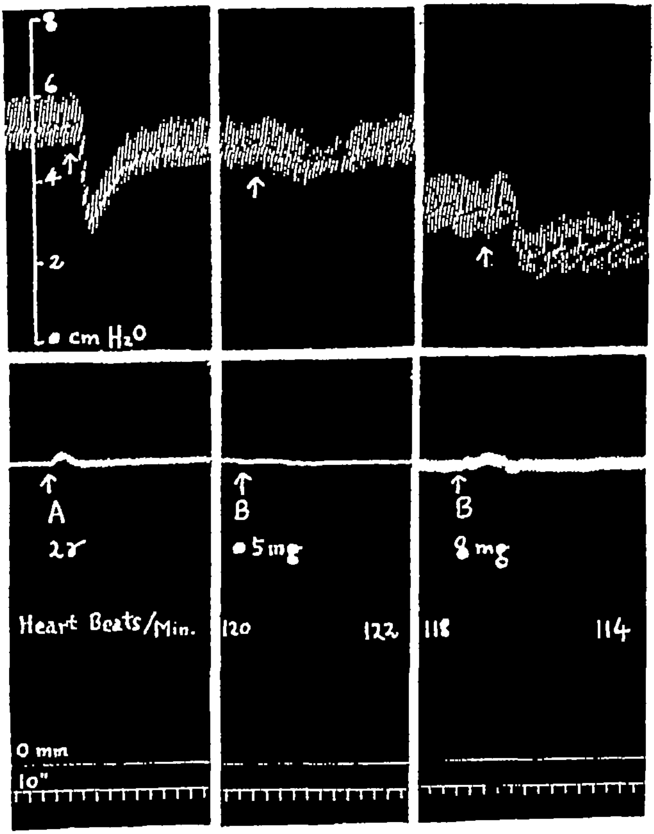


FIG 2 HEART LUNG PREPARATION FROM A DOG

Upper tracings, left auricular pressure Lower, arterial pressure *A*, adrenaline, *B*, berberine

caused a small rise of arterial pressure, a fall of left auricular pressure (fig 2), a diminution in the cardiac volume both systolic and diastolic, and a moderate increase in the coronary flow by about 30 to 50 per cent The heart rate was only slightly increased, and in an efficient heart the cardiac output was little affected Large doses (40 to 100 mgm) of berberine were, on the other

hand, definitely depressant to the heart, the left auricular pressure being raised and the rate decreased

3 Isolated heart

The isolated heart of the cat was perfused with Locke s solution by Langendorff's method. Berberine was introduced into the perfusion fluid or in jected into the rubber tubing immediately above the perfusion cannula. By perfusion berberine in concentrations 1 5×10^6 to 1 2.5×10^5 moderately augmented the contractions and increased the coronary flow by about 20 to 40 per cent. The rate was little affected In concentrations above 1 10^4 its depressant effect began to appear, contractions becoming weaker,

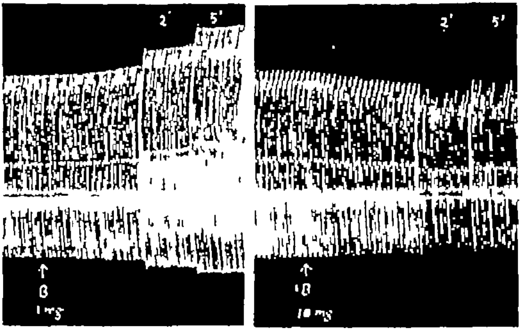


FIG 3 ISOLATED CAT'S AURICLES

Effects of small and large doses of berberine (*B*) The drum was stopped at inter vals to show the gradual effects in a small space

the heart rate slower and the coronary flow smaller But these effects were not very striking, and even when a berberine solution as strong as 1 10^4 was perfused the heart continued to beat fairly well without any sign of stopping. By injection 0 2 to 1 mgm of berberine also moderately augmented the contractions the effect being comparable to that of small doses of adrenaline.

4 Isolated auricles

The isolated rabbit s and cat s auricles were suspended in about 70 cc. of Locke s solution at a constant temperature of 28°C and with a rapid stream of oxygen bubbling through the bath. One-half to 1 mgm of berberine added to the bath augmented the contractions and large doses (5 to 10 mgm) depressed them In both cases the rate was little affected (fig 3)

5 *Tachyphylaxis*

Although it had been found that equal doses of berberine produced equal depressor effects in intact cats and dogs, tachyphylaxis was noticed in all these three heart preparations As is shown in figure 4, the second dose of berberine produced a smaller fall in the left auricular pressure of the heart-lung preparation Even in the isolated heart constantly perfused with fresh Locke's solution, an injection of a moderate dose of berberine rendered the heart less responsive to subsequent doses of the same drug

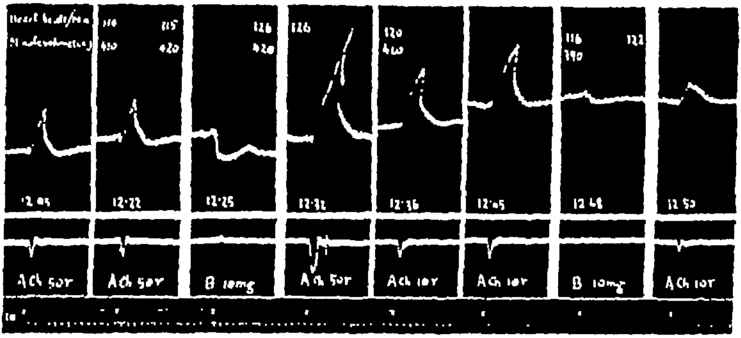


FIG 4 HEART LUNG PREPARATION FROM A DOG

Upper tracings, left auricular pressure Lower, arterial pressure *Ach*, acetylcholine, *B*, berberine

6 *Interaction with acetylcholine*

Since Marfori (quoted in Heffter's Handbuch) (2) found that a few milligrams of berberine injected into a rabbit diminished the excitability of the cardiac vagus to electric stimulation, it seemed of some interest to see if berberine exhibited the same effect on the cardiac action of acetylcholine, the humoral transmitter of the vagus To our surprise, berberine was found to possess the same biphasic effect on the acetylcholine action as we found in sympathomimetic substances in their interaction with adrenaline (14), i e , sensitisation in small doses and antagonism in larger ones On all three preparations, berberine increased and decreased the inhibitory action of acetylcholine according to its dosage or concentration

On the heart lung preparation, the first 10 mgm of berberine increased the acetylcholine effects on left auricular and arterial pressures by about five times The second dose on the other hand much diminished them (fig 4) The same biphasic action of berberine was observed in the isolated heart Two-tenths mgm of berberine much increased and 1 mgm abolished the

inhibitory effect of acetylcholine (figs 5 and 6) In the isolated auricles, small and large doses of berberine also increased and decreased or abolished the acetylcholine action respectively (figs. 7 and 8) Berberine in concentra

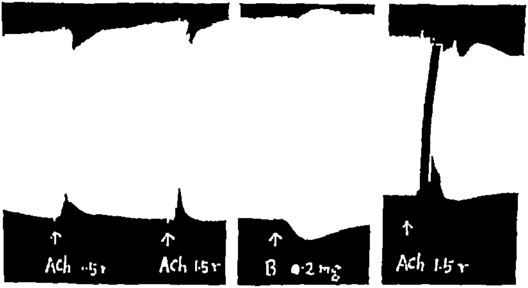


FIG 5 ISOLATED CAT'S HEART
Effects of equal doses of acetylcholine before and after a small dose of barberine (*B*)

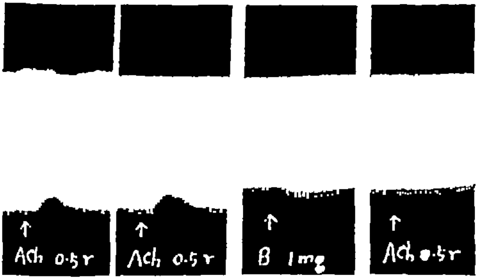


FIG 6 ISOLATED CAT'S HEART
Effects of equal doses of acetylcholine before and after a moderate dose of berberine (*B*)

tions potentiating the acetylcholine action did not affect the cardioinhibitory action of pilocarpine but did diminish or abolish the latter when the concentration was raised to 1 or 2 mgm. per 70 cc. In such high concentrations however, the inhibitory effect of KCl was often slightly increased if modified

at all The stimulant action of adrenaline was little affected by berberine in these concentrations on this preparation and on the isolated heart

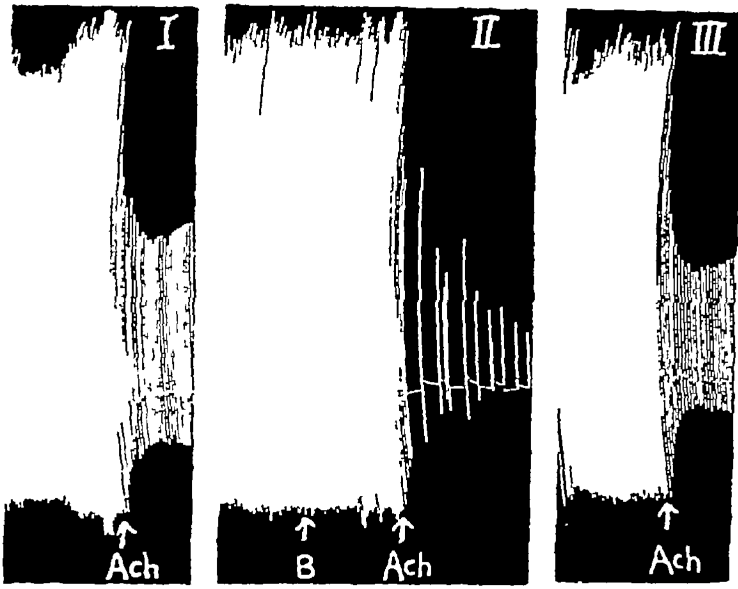


Fig 7 Isolated Rabbit's Auricles

Effects of equal doses (10γ) of acetylcholine (*Ach*) before (*I*), during (*II*), and after (*III*) a small dose (0 2 mgm in 70 cc) of berberine (*B*)

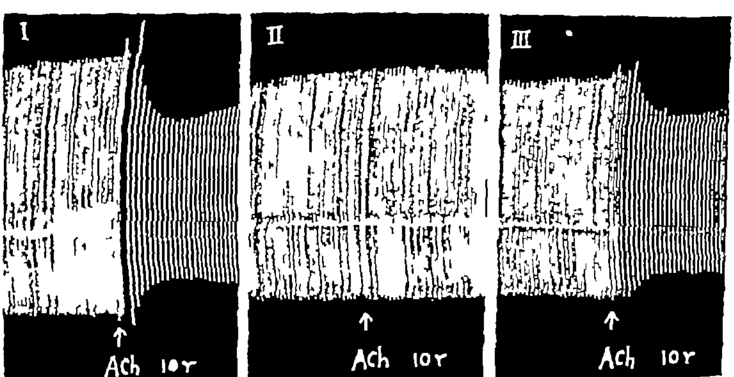


Fig 8 Isolated Cat's Auricles

Effects of equal doses of acetylcholine (*Ach*) before (*I*), during (*II*) and after (*III*) a moderate dose (1 mgm in 70 cc) of berberine (*B*)

DISCUSSION

The cardiac action of berberine

Most previous workers found that berberine is a cardiac depressant (see Chopra *et al* (7)), but Williams (15) and Soto and Sivoti (8) found that in dogs small doses of berberine stimulate the heart before depressing it. The present results have clearly shown that berberine in a fairly wide range of doses acts as a cardiac stimulant on all three preparations used. In stimulant doses berberine mainly increases the force of the contractions the rate being little affected, while depressant doses usually diminish both In the intact animals however, the cardiac action of berberine would be much modified or even completely masked by the coexisting peripheral vasodilatation, its potentiating and antagonistic actions on the vagus, and perhaps also by its central actions.

The interaction between berberine and acetylcholine

The potentiation of the cardioinhibitory action of acetylcholine by small doses of berberine appears to be a true sensitisation, certainly not a synergism, in which two agents act in the same direction In doses which cause sensitisation berberine never inhibits the heart, but often stimulates it Eserine on the other hand which also augments the action of acetylcholine, itself inhibits the heart It is also interesting to note that the eserine sensitisation though very striking on the leech muscle and the frog's rectus abdominis has never been satisfactorily demonstrated on the frog's heart, rat s gut and other smooth muscles (16, 17)

In comparison with atropine, the antagonistic action of berberine toward acetylcholine is relatively weak doses of berberine 100 to 1000 times as large as those of acetylcholine are required to effect a complete antagonism In doses antagonistic to acetylcholine, berberine also antagonises the effect of pilocarpine but not that of KCl. Since many quaternary ammonium salts are antagonistic to acetylcholine on the frog's heart and other tissues (18), this same activity may reside in its quaternary ammonium group which is closed up in the phenanthrene ring

In view of our previous findings that most of the sympathomimetic amines and their synergists and antagonists potentiate and antagonise the action of adrenaline according to their dosage or concentration the present results obtained with berberine and acetylcholine a different category of drugs are of great interest Although no generalisation is to be attempted before a more extensive survey has been made these results certainly suggest that the biphasic nature of the interaction of drugs may be much more general than has previously been suspected and may prove of great help in understanding the mode of drug action.

SUMMARY

1 The action of berberine and its interaction with acetylcholine were investigated on three mammalian heart preparations the heart-lung preparation, the isolated cat's heart and the isolated rabbit's and cat's auricles

2 Berberine stimulates the heart and augments the coronary flow in small and moderate doses, in large doses it depresses the heart

3 The cardioinhibitory action of acetylcholine is potentiated by small doses of berberine but antagonised by moderate and large doses Doses of berberine which are antagonistic to acetylcholine are also antagonistic to pilocarpine but not to KCl

The author wishes to thank Prof J H Burn for his guidance and help His thanks are also due to Dr E Bülbring and Mr H W Ling for their help in some of the experiments

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THE CIRCULATORY ACTION, AFTER ATROPINE, OF CERTAIN FURFURYL AMMONIUM IODIDES AND OF ACETYL-BETA METHYLCHOLINE CHLORIDE (MECHOLYL)[1] [2]

EDWIN J FELLOWS AND A. E. LIVINGSTON

From the Department of Pharmacology Temple University School of Medicine Philadelphia, Pennsylvania

Received for publication November 20 1940

In 1869 Crum Brown and Fraser (1) reported a curare-like action for certain quaternary ammonium bases Other effects subsequently were described (2 3 4, 5) for this class of compounds and in 1914 Dale (6) classified the actions of acetylcholine into two categories which he designated as 'muscarine" and ' nicotine-like ' In the same year Burn and Dale reported (7) that the quaternary ammonium compound Me_4N had a dual action similar to that of acetylcholine Dale (6) defined "muscarine ' action as a "peripheral action broadly reproducing the effects of cranial and sacral involuntary nerves and readily paralyzed by atropine. The term "nicotine-like ' first was used by Barger and Dale (5) to describe actions resembling those of nicotine which were exhibited by a number of quaternary ammonium com pounds Dale's criterion (6) of a ' nicotine-stimulating" action is a pressor action obtainable in pithed animals after atropine and nullified by large doses of nicotine In addition to a "nicotine-stimulating' action certain of these compounds also have a "nicotine-paralyzing' effect which is evidenced either by their interference with autonomic preganglionic conduction (5) or by their nullifying effect on the pressor action of small doses of nicotine (7)

In previous experiments (8) we found that the quaternary ammonium compounds, furfuryl trimethyl (F T M), furfuryl dimethyl ethyl (F D M E) tetrahydro-furfuryl trimethyl (T F T M) and tetrahydro-furfuryl dimethyl ethyl (T F D M E) ammonium iodides caused catharsis, increased salivary lachrymal, bronchial and gastric secretion, increased tone of intestine and urinary bladder, cardiac inhibition and a fall of blood pressure. These effects were overcome or prevented by atropine. They therefore were purely 'muscarinic However, in subsequent experiments in which we sought to determine the quantitative antagonism of atropine to the depressor action of these furfuryl compounds we found that if larger doses of both were

[1] Presented before the American Society for Pharmacology and Experimental Therapeutics Inc Toronto meeting April, 1939 (THIS JOURNAL, 66 13 1939)

[2] This investigation was supported in part by the D J McCarthy Foundation

employed pressor effects were sometimes encountered This somewhat capricious action of the furfuryl compounds, after atropine, was in marked contrast to their parasympathomimetic action which can be demonstrated in all animals before atropine The present investigation of the action of the furfuryl compounds *after atropine* has been carried out in order to determine the fact and frequency of occurrence of pressor effects as well as relative intensity of action and if possible the nature of the action responsible for it Since we had reason to suspect that the effects of these compounds, after atropine, corresponded with Dale's "nicotine-like" action, carbaminoyl choline chloride (C C, "lentin" or "doryl") and nicotine have been used as comparative agents The infrequent occurrence of the pressor action, after atropine, with the furfuryl compounds and also the large amounts of these substances required, suggested trial, under comparable conditions, of acetyl-beta-methylcholine chloride (A B M C, "mecholyl") which has been reported

TABLE 1

COMPOUND	EFFECTIVE DOSE RANGE		NUMBER OF ANIMALS USED	NUMBER OF ANIMALS IN WHICH PRESSOR EFFECTS WERE OBSERVED	RESULTS OF ALL INJECTIONS MADE	
	Cats	Dogs			Pressor	Depressor or no effect
	mgm per kgm	*mgm per kgm*				
F T M	4–30	1 5–8	57	32	149	50
T F T M	6–30	1 5–6	21	9	39	37
F D M E	30–50	2–10	20	5	17	43
T F D M E	30–50	2–10	15	4	11	38
A B M C	4–40	2–8	49	12	76	136
C C	0 5	0 1–0 2	19	19	52	4
Total			120			

by Simonart (9, 12) and Hunt (10, 11) to possess a pure "muscarine" action It was found that A B M C caused a rise of blood pressure in an occasional animal and it therefore has been included in the present studies

METHODS

Cats and dogs anesthetized with ether, nembutal or chloralosane prior to destruction of the central nervous system have been used in the present experiments All cats were pithed, decapitated, and given from 3 to 6 mgm of atropine per kilogram of body weight,[3] following which a period of one hour was allowed to elapse before any injections were made In order to determine the state of reactivity of each cat an initial test dose of 0 15 mgm nicotine was administered and was repeated until a maximum constant rise of blood pressure was obtained It was observed that too frequent injection of large doses of the furfuryl compounds sometimes resulted in a diminution of their own pressor effects after each injection In certain experiments this was attributable to

[3] Throughout the remainder of the text it is understood that all doses mentioned are per kilogram of body weight and all injections are intravenous

diminished atropine antagonism to the vasodepressor action of these compounds therefore, when extremely large doses of the furfuryl compounds were used, additional atropine was given prior to each injection of the compound under investigation Despite additional atropinization diminution in the pressor action of a test dose of nicotine by the furfuryl compounds frequently was observed In some animals this effect was slight and was present only for a few minutes but in others it was pronounced and persisted for more than an hour In cats in order to avoid complications arising from this depressant effect, the test dose of nicotine has been injected throughout an experiment, whenever necessary to determine the state of reactivity to this type of stimulation Some of the dogs used were pithed and decapitated but, most of them were pithed and not decapitated In table 1 is shown the dose range employed as well as the number of animals used and the frequency of occurrence of pressor effects.

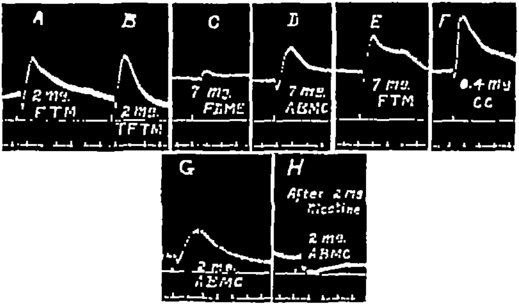

FIG 1 A and B Dog 14 kgm. pithed and atropinized (2 mgm. per kilogram)
C D E and F Dog 8 5 kgm pithed decapitated and atropinized (2 mgm. per kilogram)
G and H Dog 14 kgm, pithed, decapitated and atropinized (2 mgm per kilogram)
Upper records, carotid blood pressure by mercury manometer Signal lines indicate injections into a femoral vein Lower lines indicate time in minutes and also zero blood pressure All doses are in mgm. per kilogram

Comparative pressor activity after atropine

In our experience (8) F T M was $\frac{1}{4}$ F D M E $\frac{1}{10}$ T F T M $\frac{1}{10}$ and T F D M E $\frac{1}{150}$ as potent intravenously as C C in *depressor* action Molitor (13) has reported that intravenously A B M C and C C are equi-active depressor agents. A B M C and the furfuryl compounds manifest a weak *pressor* effect after atropine, as evidenced by our observations that in this type of action in pithed cats these compounds were $\frac{1}{25}$ as active as C C In dogs with the doses used C C appeared to be from 25 to 30 times more potent in pressor action after atropine than A B M C or any of the furfuryl compounds. F T M and T F T M are approximately equi-active pressor agents after atropine. This has been verified many times and is illustrated in figure 1

in this case 2 mgm F T M (A) caused a rise of blood pressure in a pithed, atropinized dog which is comparable with that produced by the same dose of T F T M (B) F D M E and T F D M E also were found to be equi-active pressor agents after atropine Saturation of the furane ring of F T M and F D M E therefore has reduced their depressor activity 10 and 6 fold respectively but has not altered their pressor action after atropine F D M E and T F D M E always were found to be considerably less potent in their pressor action after atropine than the trimethyl compounds and A B M C, as illustrated in figure 1 where 7 mgm F D M E (C) produced a much smaller rise of pressure than the same amount of A B M C (D) and also F T M (E) It is to be noted that the illustrated pressor action of A B M C (D) is of approximately the same intensity as that of F T M (E) This is typical of other experiments carried out on 10 cats and dogs The much greater pressor potency, after atropine, of C C is illustrated in figure 1 where 0 4 mgm C C (F) caused a much greater rise of pressure than that produced in the same animal by 7 mgm of A B M C (D) and 7 mgm of F T M (E)

Effect of a paralyzing dose of nicotine on pressor action after atropine

It has been found in 3 pithed animals in which pressor responses, after atropine, have been established with F T M, T F T M, F D M E, T F D M E, and A B M C, that a paralyzing dose of nicotine converts pressor into depressor effects This is illustrated in figure 1 by A B M C where 2 mgm of this compound in a completely pithed, atropinized dog caused a pressor effect (fig 1, G) but after a paralyzing dose of nicotine (2 5 mgm) injection of 2 mgm of A B M C caused a fall of blood pressure (fig 1, H) The furfuryl compounds and A B M C therefore cause pressure effects by a peripheral mechanism since a rise of blood pressure may be demonstrated after destruction of the central nervous system, it is probable that these effects are a manifestation of Dale's "nicotine-stimulating" action because they are demonstrable before but not after doses of nicotine sufficient to paralyze ganglia

Effect of large doses of the furfuryl compounds and A B M C on the pressor action of small doses of nicotine (nicotine-paralyzing action)

Figure 2 (A, B and C) shows that successive injections of the test dose of nicotine employed in the present experiments give no indication of diminished pressor activity in an atropinized cat This indicates that the test dose of nicotine used is stimulant in its action and devoid of paralyzing effects That large doses of the furfuryl compounds and A B M C exhibit "nicotine paralyzing" properties is illustrated in figure 2 In an experiment on a cat which had previously received 4 mgm of atropine sulfate, a test dose of nicotine produced a marked pressor effect (fig 2, D) Immediately after this, 30 mgm of F T M produced a rise of blood pressure preceded by a slight fall (E) Injection of the same amount of nicotine which produced

the rise of pressure at D now shows that the animal is almost entirely unresponsive to this agent (F) At the end of 40 minutes (G) the animal almost had returned to its normal control reactivity (D) to nicotine stimulation An additional 1.25 mgm. of atropine was given followed by 30 mgm. of A B M C and a rise of pressure obtained (H) which is not preceded by the primary fall of pressure that was observed with F T M (E) The absence of a preliminary fall of pressure is probably due to more complete suppression

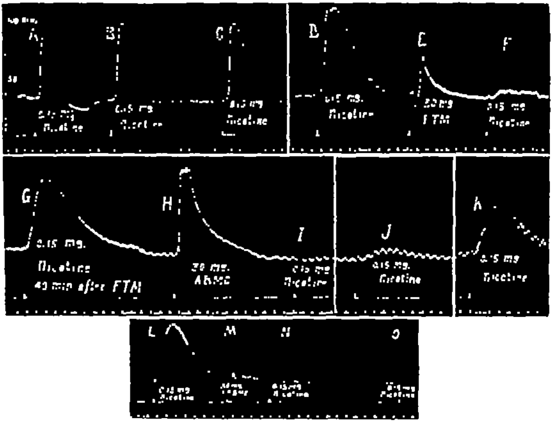

FIG 2 A, B and C Cat 2.8 kgm pithed, decapitated and atropinized (5 mgm. per kilogram)
D to K Cat 3 kgm. pithed decapitated and atropinized (5 mgm per kilogram)
L, M N and O Cat 3.3 kgm. pithed, decapitated and atropinized (5 mgm. per kilogram)
Upper records carotid blood pressure by mercury manometer Signal lines indicate injections into a femoral vein and also zero blood pressure Lower lines indicate time in minutes. All doses are in mgm per kilogram of body weight.

of depressor action by the additional atropine administered just prior to A B M C The nullified pressor action of the test dose of nicotine (I) as compared with the rise obtained prior to A B M C (G) demonstrates that A B M C also has depressed the animal to this type of stimulation. Marked depression to nicotine stimulation is still apparent (J) after 35 minutes and even after 70 minutes (K) nicotine pressor action had not entirely assumed its control effectiveness (G) The trend of these results is typical of 9 other experiments carried out on 4 cats

In a cat in which a test dose of nicotine produced a considerable rise of blood pressure (fig 2, L) the injection of 30 mgm of T F D M E caused a scarcely perceptible rise of pressure (M) Four minutes later the pressor action of the test dose of nicotine is shown to be completely nullified (N) and the state of complete unresponsiveness to nicotine stimulation is shown (O) still to be present 25 minutes after administration of T F D M E This illustrates results which have been obtained with 10 other cats, indicating that while a furfuryl compound may cause little if any change in blood pressure when injected, the animal may react less readily or may be entirely unresponsive to subsequently administered ganglionic stimulants

Effect of adrenalectomy on pressor action after atropine

Dale (6) has reported that adrenalectomy lessens the pressor action, after atropine, of certain choline derivatives, and Burn and Dale (7) have made the same observation in the case of Me_4N In the present experiments it has been found that established pressor effects are reduced but still present after adrenalectomy in the case of F T M, T F T M, T F D M E and F D M E In 4 experiments it was found that the pressor action of A B M C was reversed by adrenalectomy as illustrated in figure 3, where 4 mgm A B M C is seen to have caused a rise of pressure (A) in a pithed, decapitated, atropinized cat After adrenalectomy the 4 mgm dose of A B M C caused only a slight fall of pressure (C) Recently Koppanyi, Linegar and Herwick (14) have reported that the pressor action of acetylcholine is not diminished by adrenalectomy if blood pressure is maintained by acacia infusion In the present experiments no attempt was made artificially to maintain blood pressure but as illustrated in figure 3 the blood pressure level was not materially reduced after adrenalectomy (C compared with A) The inconsistency of A B M C action, after atropine, has precluded an extensive investigation of its pressor action, but it would appear that under the conditions of the present experiments this action is due largely to activation of the adrenal medulla In figure 3 it is shown that in the same animal in which A B M C pressor effects, after atropine, were nullified by adrenalectomy the marked pressor action of the 0 15 mgm dose of nicotine before (B) is materially reduced after (D) adrenalectomy

Potentiation of pressor action, after atropine, by eserine

Since pressor effects, after atropine, were not demonstrable with the furfuryl compounds and A B M C in all animals (table 1) a number of eserinized cats and dogs were used in an attempt to secure a preparation in which pressor effects could be more consistently duplicated While a more consistent preparation was not obtained it was found that in certain cases eserine did potentiate the pressor action of F T M and A B M C In figure 3 is shown a typical example of 4 experiments Four milligrams A B M C caused a rise

of pressure (fig 3 E) in an atropinized cat. Five-tenths milligram eserine next was injected and the animal then given 4 mgm. of A B M C and it is to be noted that the rise of pressure produced (fig 3, F) is very decidedly greater than that obtained with this dose prior to eserine (E) That the pressor action of F T M, after atropine, also may be potentiated by eserine

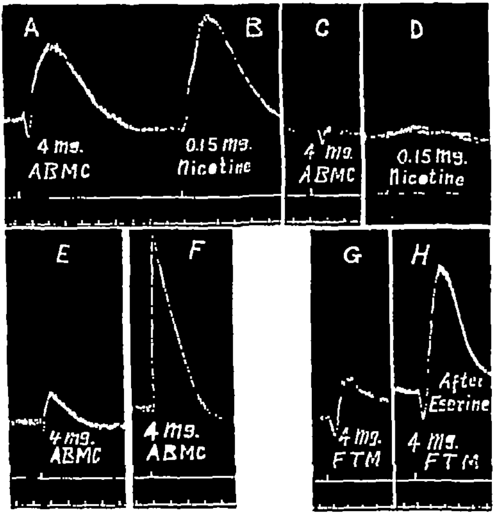

FIG 3 A B C D Cat 3.8 kgm., pithed decapitated and atropinized (4 mgm. per kilogram) A and B before C and D after adrenalectomy E, F Cat 4 kgm, pithed decapitated and atropinized—E before F after eserine G and H Dog 24 kgm. pithed and atropinized (2 mgm per kilogram) Upper records femoral blood pressure by mercury manometer Signal lines indicate injections into a femoral vein. Lower lines indicate time in minutes and also zero blood pressure All doses are in mgm per kilogram of body weight.

is illustrated in figure 3 where 4 mgm F T M caused a rise of pressure (G) in a pithed decapitated atropinized dog. The same dose of F T M after 0 1 mgm eserine produced a much greater rise of pressure (H) In these experiments it was observed that the degree of potentiation of pressor action usually was not as great in the case of F T M as with A B M C

DISCUSSION

From the present experiments it is apparent that F T M, T F T M, F D M E, T F D M E or A B M C have a mild "nicotine-like" action when compared with such compounds as C C or nicotine itself The doses of the furfuryl compounds and A B M C required to demonstrate the "stimulating" fraction of their "nicotine-like" action are so large that "nicotine-paralyzing" effects also may be observed In our experience a "nicotine stimulating" action was demonstrable more frequently with the furfuryl compounds and A B M C in those cats in which marked pressor effects were obtained with small doses of nicotine The results obtained with eserinized animals in the present experiments are another indication that one of the most important factors in the demonstration of "nicotine-stimulating" properties for these compounds is the state of vasoconstrictor or vasodilator reactivity of the animal used It was found that while eserine may increase the magnitude of pressor action of the furfuryl compounds and A B M C in those animals *responding with a rise of pressure* it did *not* cause those animals *responding with depressor effects* with the range of doses used *to show pressor effects* It would appear, therefore, that in certain animals the vasodepressor action of the furfuryl compounds and A B M C is so overwhelmingly preponderant that its suppression by atropine is not sufficiently complete to permit unmasking of the mild vasopressor action This observation has been made even for acetylcholine, a much more potent pressor agent after atropine, by Linegar, Herwick and Koppanyi who report (15) that in certain animals even massive doses (30 to 40 mgm) of atropine failed to abolish completely the vasodilator effect of large or even moderate doses of acetylcholine

While a more consistent "nicotine-stimulating" preparation was not obtained the potentiation of established pressor action of F T M or A B M C by eserine is of interest from the standpoint of the mechanism of action of the latter agent In the case of F T M, augmentation of pressor action, after atropine, must be explained on some basis other than inhibition of esterase because it has been found (8) that the furfuryl compound is stable in blood It also is probable that potentiation of the pressor action of A B M C, after atropine, by eserine is not entirely explained by inhibition of cholinesterase While A B M C is labile in blood its destruction is decidedly less rapid than that of acetylcholine In our experience (8) a period of 10 to 12 minutes contact with blood was required to completely inactivate 0 001 mgm of A B M C Destruction of at least several thousand times this amount in a few seconds must be inferred at figure 3, E, to explain the augmentation of A B M C pressor action at figure 3, F, after atropine, by eserine on the basis of cholinesterase inhibition Evidence of an augmentary action by eserine which is not referable to its anti-cholinesterase effect also has been observed by Feldberg and Vartiainen (16) who reported that the

ganglion stimulating action of choline, potassium ions nicotine and hordenine methiodide was potentiated by eserine and therefore concluded that the latter agent may cause a non-specific sensitization of ganglion cells to a variety of chemical stimuli.

As previously mentioned Simonart (9, 12) and Hunt (10 11) have reported that A B M C is devoid of 'nicotine-like' properties In the present experiments as shown in table 1, "nicotine-stimulating" effects were obtained with A B M C in pithed cats after atropine, in doses of from 4 to 40 mgm It must be stated however, that our effective dose range usually was of the order of 30 mgm While it has been reported by Simonart (9) that A B M C does not produce a rise of blood pressure in pithed cats *in any dose* as far as we could determine the largest dose used by this author was of the order of 1 5 mgm It is conceivable that the materially larger amounts of A B M C and atropine used in our experiments may account for the difference in the present report and that by Simonart (9) Even in the relatively low dose range used, Simonart reported that A B M C caused a slight stimulant effect on the denervated gastrocnemius muscle of the cat. In discussing the 'muscarine-like' and "nicotine-like' actions of Me_4N Burn and Dale (7) have reported— "it is easy to show that in cases showing a pure depressor effect under anesthetic one can obtain a practically pure pressor effect in the same animal after complete pithing of the central nervous system " Hunt's statements (10 and 11) to the effect that A B M C is devoid of "nicotine-like effects are based on experiments (17) in which A B M C always produced a fall of blood pressure after atropine Why we obtained pressor effects with A B M C after atropine, and Hunt did not is probably best rationalized by the fact that all of our experiments were *carried out on animals with central nervous system destroyed* whereas Hunt's were *carried out on animals with central nervous system intact.*

SUMMARY

1 In cats and dogs with central nervous system destroyed it was found that certain doses of furfuryl trimethyl (F T M) furfuryl dimethyl ethyl (F D M E), tetrahydro-furfuryl trimethyl (T F T M) and tetrahydro-furfuryl dimethyl ethyl (T F D M E) ammonium iodides and also acetyl-beta methyl choline chloride (A B M C) after atropine may cause a rise of blood pressure which is reversed by a paralyzing dose of nicotine

2 The pressor action of F T M, T F T M and A B M C, after atropine was found to be of approximately equal intensity All three are much less potent in their pressor action after atropine, than carbaminoyl choline chloride (C C) F D M E and T F D M E are less active pressor agents after atropine than F T M T F T M or A B M C

3 F T M T F T M F D M E, T F D M E or A B M C have a "nicotine paralyzing action as indicated by a nullifying effect of large amounts of these compounds on the pressor action of small doses of nicotine

4 After adrenalectomy pressor effects, after atropine, are somewhat reduced in the case of F T M, F D M E, T F T M and T F D M E With the doses of A B M C used the pressor action after atropine, was entirely nullified after adrenalectomy

5 When pressor effects, after atropine, were established with F T M or A B M C, eserine potentiated the pressor action of these compounds Because of its stability in blood, F T M potentiation by eserine must be explained on some basis other than esterase inhibition and it is doubtful if this mechanism of action entirely explains augmentation of the pressor action, after atropine, of A B M C

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THE TOXICITY OF DILAUDID INJECTED INTRAVENOUSLY INTO MICE

M. E. BUCHWALD AND G S EADIE

From the Department of Physiology and Pharmacology Duke University School of Medicine, Durham, N C

Received for publication October 17 1940

The data in the literature on the toxicity of dilaudid on intravenous administration are scanty Umezato (1) gives 60 mgm per kilogram as the minimal lethal dose for mice as compared with 300 mgm. per kilogram for morphine. Eddy and Reid (2) find that the average fatal dose subcutaneously is 84.26 mgm. per kilogram of the base (equivalent to 95 04 mgm. per kilogram of the hydrochloride), and that it is six times as toxic as morphine The older literature is reviewed by King Himmelsbach and Sanders (3) the toxicity relative to morphine has been reported all the way from 1 1 (rabbit) to 5 1 (frog)

EXPERIMENTAL

The data presented were obtained from the following experiments *Dilaudid I* A series of mice weighing about 16 grams (average 16 4) were injected with dilaudid hydrochloride[1] over the period July 18 to July 28 1939 They were divided into 11 groups of nine mice each, receiving 11 different dosages *Dilaudid II* A second series of 57 mice of the same strain (average weight 14 6 grams) was injected with dilaudid hydrochloride on June 2 1940 They were divided into approximately three equal groups and three different doses were used *Morphine I* At the same time as the preceding another series of 56 of the same mice was injected in a similar way with three different doses of morphine hydrochloride. *Dilaudid III* On September 24, 1940 a group of 99 mice of a different strain (average weight 21.8 grams) was injected with dilaudid hydrochloride in a similar way to the first dilaudid series and, *Morphine II*, at the same time another group of 99 mice of the same strain was injected with morphine hydrochloride. The animals of the dilaudid II and morphine I series as well as those of the dilaudid III and morphine II series, were kept under identical conditions

A freshly prepared solution of the drug was injected into the tail vein

[1] We are indebted to the Bilhuber Knoll Corporation for the gift of 1 gram of dilaudid.

Although most of the deaths occurred within two or three hours, the final count was not made before 24 hours The other experimental details and the results are given in the accompanying table

The data have been treated by the statistical methods of Bliss (4), using the tables of Fisher and Yates (5), the symbols used are those employed by these authors Briefly the process consists of fitting the data, properly weighted, to the probability curve When this is done, and y, the mortality

TABLE 1

	DILAUDID						MORPHINE				
	I		II		III			I		II	
Dose	Total number of mice	Number of survivors	Total number of mice	Number of survivors	Total number of mice	Number of survivors	Dose	Number of mice	Number of survivors	Number of mice	Number of survivors
mgm per kgm							mgm per kgm				
30	9	9			9	8	225	9	3		
40	9	8			9	5	250	9	4		
50	9	7	19	16	9	6	275	9	4		
60	9	7			9	4	300	9	5	19	11
70	9	5			9	7	310	9	1		
80	9	6	20	13	9	4	320	9	3		
90	9	3			9	2	330	9	4		
100	9	5			9	1	340	9	1		
110	9	1	18	7	9	2	350	9	2	20	8
120	9	2			9	0	375	9	2		
130	9	0			9	0	380			17	2
							400	9	0		
$\bar{x}$	1 9007		1 8957		1 5587			2 5273		2 4932	
$\bar{y}$ (= a)	5 001 ±0 0279		4 6896 ±0 0064		5 3018 ±0 0217			5 2983 ±0 0390		5.5420 ±0 0157	
b	5 0239 ±1 0743		3 7385 ±0 0364		3 9514 ±0 6528			11 9288 ±2 3191		4 0609 ±3 6478	
log	1 9005 ±0 0282		1 9818 ±0 0487		1 7824 ±0 0377			2 5022 ±0 0177		2.3547 ±0 0699	
L D.50	79 5		95 9		60 6			317 8		226 3*	

* In milligrams per kilogram.

in probits, is plotted against x, the dose in logarithms, a straight line is obtained (fig 1) The equation of this line is

$$y = a + b\,(x - \bar{x})$$

$\bar{x}$ being the weighted average log dose $\bar{y}$, the weighted average probit, is the corresponding value of y, and is numerically equal to a The constants for each equation are given in the table

That the use of the probability curve is justified can be shown by plotting the experimental points show no trend towards anything but a straight line, and this is confirmed by the χ^2 test This is satisfactory for all five curves and gives no indication of departure from the theoretical equation The standard error of the 50 per cent death point is given in the table as well as

the standard errors of the two constants of the equation, a and b These last were determined from the equations

$$V(a) = \frac{\chi^2}{nS(w)}$$

and

$$V(b) = \frac{\chi^2}{nA}$$

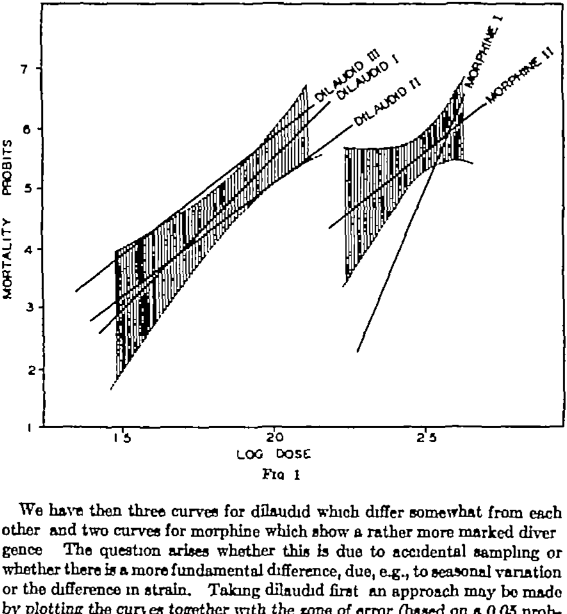

FIG 1

We have then three curves for dilaudid which differ somewhat from each other and two curves for morphine which show a rather more marked divergence The question arises whether this is due to accidental sampling or whether there is a more fundamental difference, due, e.g., to seasonal variation or the difference in strain. Taking dilaudid first an approach may be made by plotting the curves together with the zone of error (based on a 0.05 probability) of one of them When this is done it is found that dilaudid II lies within the zone of error of dilaudid I and that dilaudid III does not It seems

likely from this that the difference between curves I and II is due to sampling, and that III diverges for other reasons This is confirmed by calculating the value of t with respect to the two constants of the equation Comparing curves I and II we find $t(a)$ 1 79 which gives (n = 10) P between 0 2 and 0 1 Similarly $t(b)$ is 1 22 and P between 0 3 and 0 2 There is therefore no indication of a difference other than in sampling When we come, however, to the comparison of curve III with these two, we find that $t(a)$ for I and III is 2 17 which for n = 18 gives P between 0 05 and 0 02, and $t(a)$ for II and II is 5 13 with P less than 0 001 (n = 10) There has been, therefore, a real shift in the value of the constant a With respect to the constant b, for curves I and III $t(b)$ is 0 82, and P between 0 4 and 0 5 (n = 18), for curves II and III it is 0 26, and P lies between 0 8 and 0 9 (n = 10) There is then no indication of a significant difference in the slope of the curves Hence we may say that these curves are probably parallel and will differ only in shifting up and down the y-axis

Since the first and second dilaudid curves differ only in sampling, we may combine them This gives us a value for a (or $\bar{y}$) or 4 8793 and for b of 4 6343 The logarithm of the estimated 50 per cent death point is 1 9259 ± 0 0242, or a dosage of 84 3 mgm per kilogram with a range of 79 8 to 89 2 This value may be compared with the average fatal dose of 95 mgm per kilogram given by Eddy and Reid The third curve gives the logarithm of L D 50 1 7824 ±0 0377 or 60 6 mgm per kilogram, with a range of 55 6 to 66 1 and it is evident that this group (of somewhat heavier mice) was, under the conditions of the experiment, more susceptible With the present data it is impossible to be more precise about the cause of this increased susceptibility, but we hope to investigate this point in future work

On applying the t test to the morphine curves we find, on the other hand, that $t(a)$ is 1 84 and P between 0 1 and 0 05, while $t(b)$ is 5 34 and P between 0 05 and 0 02 (n = 10 in both cases) The shift here has been in the value of b, the slope, rather than in a

It is customary to compare the toxicity of morphine with that of dilaudid To do this it is necessary to choose points of equal effect If we choose the 50 per cent death point, we have, for dilaudid I, dilaudid II and the combined curve compared with morphine I, and for dilaudid III compared with morphine II the following dose ratios respectively 4 0, 3 3, 3 8 and 3 7 These values are somewhat lower than those of Umezato and of Eddy and Reid It is interesting to note that the value for dilaudid III and morphine II, 3 7, is very close to 3 8 obtained from the combined dilaudid I and II curves and morphine I (These particular combinations were chosen because the data were obtained from the same strain, and for the most part simultaneously)

How far this ratio will hold, however, when a different point of comparison is chosen, is another question Graphically, the answer is easy to see the logarithm of the ratio of the two doses is the horizontal distance between the

two curves at points corresponding to the same value of y The ratio will remain unaltered only if the dilaudid curve is parallel to the morphine curve For morphine II this condition is very nearly fulfilled indeed a sampling error might be expected to account for a greater difference in slope than that observed With morphine I the results are different the ratio increases considerably as the point chosen for comparison represents a smaller and smaller percentage mortality For example taking the combined I and II dilaudid curves and morphine I, we find for 50 per cent mortality a ratio of 3.8, for 1 per cent 7 6 while for a mortality of 0 01 per cent the ratio would rise to 11 7 These figures are, of course subject to the error involved in the determination of the b value for each separate curve It may be recalled that Umezato using the minimum lethal dose which is lower than the 50 per cent death point got a ratio of 5, rather than 3 to 4

It is impossible, therefore in cases like this to make any precise statement about the relative toxicity without fixing the expected percentage mortality

That this sort of phenomenon is not confined to toxicity is indicated by the work of Schoen (6) He noted in rabbits that dilaudid is five times as effective as morphine in depressing respiration for which relatively small doses are required, but when larger doses which stimulate respiration are used, it is only twice as strong The size of the dose, however, is apparently not the only factor, for in investigating toxicity he finds the minimal effective dose to be 5 mgm per kilogram for morphine and 0.5 mgm per kilogram for dilaudid, a ratio of 10 1 On respiration on the other hand, he finds a ratio of 5 1 over a dose range of 1 to 10 mgm per kilogram.

There is no reason to believe that similar variations of toxicity and other effects with dosage will not be found for other drugs since the chances that two straight lines obtained in this way will be parallel must be very small

SUMMARY

The intravenous toxicity of dilaudid for mice has been determined on three separate occasions using 99, 57 and 99 mice respectively The L.D 50 was found in two instances to be 80 and 96 mgm. per kilogram there being no significant difference between these figures In the third instance it was 61 mgm. per kilogram a difference probably to be attributed to a difference in season and in strain Two similar determinations were made for morphine the L.D 50 was found to be 318 and 226 mgm. per kilogram a significant difference and due probably to the same reasons. At the 50 per cent death point dilaudid is 3 7 to 3.8 times as toxic as morphine. In the pair of curves made in the autumn this value is maintained fairly closely throughout all dose ranges, but in the pair of curves made in the summer this ratio increases with decreasing mortality reaching 11 7 at an expected mortality of 0 01 per cent It is therefore necessary to take the expected mortality into account when comparing the toxicity of two drugs Similar considerations probably apply to other effects of these drugs.

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EFFECT OF SULFUR COMPOUNDS IN THE DIET ON SULFANILAMIDE CYANOSIS AND ANEMIA

ARTHUR P RICHARDSON

From the Department of Pharmacology Stanford University School of Medicine San Francisco Calif

Received for publication October 23, 1940

The cyanosis which occurs during therapy with sulfanilamide ordinarily causes no concern because it is usually associated with the formation of only methemoglobin. Occasionally however sulfhemoglobin is encountered, and because of the extreme stability of this pigment, its occurrence is regarded by some to be a serious complication of bacterial chemotherapy with sulfanil amide. Comparatively little is known of the mechanisms underlying the formation of sulfhemoglobin The pigment has been described as resulting from the administration of a variety of aniline derivatives and is also supposed to occur for unknown reasons, in cases of so called 'enterogenous cyanosis' (1)

Snapper administered phenacetin to dogs and showed that sulfhemoglobin was formed only when a large quantity of sulfur was given at the same time (2) The logical deduction from Snapper's work is that sulfhemoglobinemia in human patients from sulfanilamide might be prevented by limiting the sulfur content of the diet (3) However there is little agreement as to how this should be done. For example, magnesium sulfate as a cathartic is considered by many clinicians to be contraindicated while sulfanilamide is being used. On purely theoretical grounds it is difficult to see how even an excessive amount of inorganic sulfate could be utilized directly in the production of sulfhemoglobin. Indirectly it is possible that catharsis might be responsible for decomposition of food and sulfide-formation due to premature movement of contents into the lower intestine (3) however no evi dence in support of this possibility exists.

The production of sulfhemoglobinemia and anemia in mice fed a diet of Purina Dog Chow containing sulfanilamide (4 5) has furnished a convenient means of studying these toxic reactions under controlled conditions. This report presents the results of experiments in which different types of sulfur compounds were tested for their ability to produce sulfhemoglobin when administered together with sulfanilamide The possible relation of changes in hemoglobin to production of anemia is also considered

METHODS

The Purina Dog Chow diet used, as in previous work on sulfanilamide with mice (4, 5), had a fairly high sulfur content (0.23 per cent total sulfur, of which 0 15 per cent was in organic combination) (6) In order to determine whether this amount of sulfur was an important factor in the production of cyanosis, we first fed sulfanilamide in other diets containing minimal concentrations of sulfur Of a number of synthetic diets tried, the one used by Addis[1] for the study of protein metabolism in rats was found to be the most satisfactory Although no chemical analyses were made, it was obvious from a study of the constituents that it contained a minimum of sulfur Two per cent sulfanilamide in this diet fed to mice for a period of two weeks produced only a very slight cyanosis or sulfhemoglobinemia This diet was therefore satisfactory as a basis to which various sulfur compounds could be added in order to see which ones could be utilized in the formation of sulfhemoglobin By altering the diet and producing varying degrees of cyanosis, we also could determine the relation, if any, between changes in hemoglobin and blood destruction

The procedure was as follows After control observations of hemoglobin and reticulocytes, groups of 6 to 8 mice were given the basic low sulfur diet to which various sulfur compounds were added, usually in high enough concentration to bring the sulfur content of the diet up to 0 25 per cent, or approximately equivalent to that of Purina Dog Chow Some groups of mice were given the sulfur diets plus 2 per cent sulfanilamide[2] while other groups, serving as controls, received only the sulfur-containing diets After two weeks hemoglobin and reticulocytes were determined again, following which the mice were bled by cardiac puncture, and the blood from each group of animals was pooled In this composite sample oxyhemoglobin was determined in terms of oxygen capacity by the method of van Slyke (7) Total hemoglobin was determined as blood iron by the method of Coombs (8) Spectroscopic analysis was carried out according to methods previously described (4), and blood sulfanilamide content was determined by the method of Marshall (9) The following sulfur compounds were tested thiourea, magnesium sulfate, sodium thioglycollate, cysteine hydrochloride, sodium thiosulfate, washed flowers of sulfur, and sulfurated potash (U S P)

Influence of sulfur compounds on cyanosis

The effects of the seven different types of sulfur compounds on the formation of sulfhemoglobin are presented in table 1 The per cent of inactive hemoglobin was calculated as the difference between the values for blood iron and oxygen capacity, divided by the blood iron Previous studies (4) have shown that determination of inactive hemoglobin pigment by this method is the most accurate means of detecting formation of methemoglobin or sulfhemoglobin

It is seen that magnesium sulfate and thiourea had no effect on the formation of sulfhemoglobin, there being no more inactive hemoglobin when these compounds and sulfanilamide were added to the diet than when sulfanilamide alone was added Sodium thioglycollate and cysteine hydrochloride both

[1] This diet contained corn meal 68 per cent, linseed oil 10 per cent, dried alfalfa 2 per cent, powdered casein 10 per cent, lard 5 per cent, cod liver oil 3 per cent, bone ash 1 5 per cent and sodium chloride 0 5 per cent

[2] The sulfanilamide was kindly furnished by Abbott Laboratories, North Chicago, Illinois

appeared to increase the cyanosis slightly and more sulfhemoglobin was present in the blood. Sodium thiosulfate, powdered sulfur and sulfurated

TABLE 1

Effect of sulfur compounds on sulfanilamide cyanosis and anemia in white mice

SULFUR COMPOUND ADDED TO BASIC DIET	ADDED SULFUR	NUMBER OF MICE USED	BLOOD SULFANILAMIDE Free	BLOOD SULFANILAMIDE Total	SPECTROSCOPIC SULFHEMOGLOBIN	INACTIVE HEMOGLOBIN	AVERAGE CHANGE IN HEMOGLOBIN*	AVERAGE CHANGE IN RETICULOCYTE*
Controls								
	per cent		mgm. per cent	mgm. per cent		per cent	grams per cent	per cent
Basic diet	0 00	6			(−)	0 2	−0 30 ±0 37	−1 0 ±0 70
2 per cent thiourea.	0 25	7			(−)	0 1	−0 04 ±0 31	−0 6 ±0 60
0 75 per cent $MgSO_4$	0 25	8			(−)	0 0	−0 43 ±0 48	−0 18 ±0 54
0 9 per cent cysteine HCl	0 25	8			(−)	1 2	+0 34 ±0 28	−0 29 ±0 50
Albumin replacing casein	0 10	7			(−)	0 0	−0 04 ±0 37	−0 34 ±0 41
0 75 per cent Na thiosulfate	0 25	16			(±)	1 1	−0 10 ±0 03	−0 35 ±0 67
0.25 per cent sulfur	0 25	8			(±)	2 8	+0 06 ±0 15	+0 51 ±0 41
0.5 per cent poly sulfide	0 10	6			(±)	1 7	−0 30 ±0 49	+0 3 ±0 86
Two per cent sulfanilamide								
Basic diet	0 00	14	16 4	19 5	(±)	4 9	−3 2 ±0 81	+19 1 ±2 9
2 per cent thiourea.	0 25	14	16 7	18 9	(±)	5 7	−6 9 ±0 68	+28 1 ±3 1
0 75 per cent $MgSO_4$	0 25	13	17 8	21 1	(±)	4 7	−4 0 ±0 87	+15 4 ±0 9
0 9 per cent Na thioglycollate	0 25	9	20 3		(+)	8 5	−4 2 ±0 60	+15 9 ±1 7
Albumin replacing casein	0 10	8	19 9	23 6	(+)	8 2	−4 3 ±0 80	+20 6 ±1 6
0.9 per cent cysteine HCl	0 25	7	18 9		(+)	10 9	−5 4 ±0 74	+16 4 ±1 9
0 75 per cent thiosulfate	0 25	16	16 5	19 4	(++)	16 2	−5 4 ±0 75	+26 2 ±2 1
0.25 per cent sulfur	0 25	13	18 0	21 3	(++)	15 2	−4 7 ±0 44	+19 5 ±2 8
0.5 per cent poly sulfide	0 10	15	15 9	20 4	(+++)	22 2	−4 8 ±0 42	+26 4 ±2 4

* Mean results with standard error of the mean

potash (potassium polysulfide) all increased the amount of sulfhemoglobin when these compounds and sulfanilamide were added to the diet. With the polysulfide the effect was particularly striking since the animals receiving

this type of sulfur became markedly cyanotic within a few days and remained so for the period of the observation

In order to explain the cyanosis produced, when animals were fed Purina Dog Chow we modified the basic diet so that it contained sulfur compounds in a form which might occur naturally in food For this purpose the casein of the Addis diet was replaced by egg albumin which is fairly rich in organic sulfur The result was that the sulfanilamide added to this modified diet produced a moderate increase in amount of sulfhemoglobin

Sulfur compounds alone added to the diet caused no changes, except in the mice fed sodium thiosulfate, sulfur and potassium polysulfide In these mice the blood was slightly darker than normal, and when concentrated specimens were examined spectroscopically, there was a faint band characteristic of sulfhemoglobin However, there was only a doubtful increase in inactive hemoglobin

The results obtained confirm the view that an increased intake of certain types of sulfur compounds in the diet leads to an increased production of sulfhemoglobin by sulfanilamide However, only the more labile sulfur compounds are effective, the more stable compounds like magnesium sulfate, and thiourea being totally inactive

Influence of sulfur compounds on anemia

Previous work on the effect of sulfanilamide on the blood of mice (10) showed that the most satisfactory indication of blood damage by this drug could be obtained from changes in hemoglobin and reticulocytes Both determinations were made in this study as they give information on different reactions, i e , changes in hemoglobin afford an estimate of blood destruction, while reticulocytes give an index of the ability of the bone marrow to compensate for the blood destruction For the sake of brevity, the difference in values before and after medication were calculated for each mouse, and the mean change for each group of mice recorded in the Table

There was no obvious relation between the amount of inactive hemoglobin and the degree of anemia The greatest loss of hemoglobin occurred in the group of animals receiving thiourea, but in these animals only a minimal amount of sulfhemoglobin was formed On the other hand, mice receiving polysulfides and sulfanilamide became very cyanotic, but no more anemic than did the slightly cyanotic animals Clearly, therefore, the formation of sulfhemoglobin in itself was not necessarily related to blood destruction

Changes in reticulocyte percentages generally paralleled the decrease in hemoglobin, thus indicating that there was no demonstrable depression of the bone marrow

Control groups of mice fed the various sulfur compounds without sulfanilamide showed no significant changes in either hemoglobin or percentage of reticulocytes

Effect of feeding sodium nitrite on blood

Since blood destruction could occur without the formation of appreciable amounts of sulfhemoglobin it seemed worth while determining if the two effects on blood could not be further dissociated, that is, whether changes in pigment might not occur without the development of anemia. For this purpose sodium nitrite was administered in Purina Dog Chow to groups of mice for two weeks and their blood was studied according to the methods already described. Concentrations of the drug up to one per cent produced little effect but with from one to four per cent the mice became moderately cyanotic and determinations of oxygen capacity and blood iron revealed inactive hemoglobin of from 11.5 to 15.5 per cent. Spectroscopic examination showed a broad absorption band at 630 mμ which readily disappeared on the addition of cyanide and hydrosulfite indicating the presence of methemoglobin. In contrast to the results obtained with the administration of sulfanilamide in the same diet there was no demonstrable sulfhemoglobin. To test further the effect of nitrite on hemoglobin this drug was fed to mice in a diet containing three times the amount of flowers of sulfur necessary to produce a marked sulfhemoglobinemia with sulfanilamide. Even under these conditions nitrite caused cyanosis associated only with methemoglobinemia.

These results demonstrate a striking difference in the action of sulfanilamide and of nitrite on hemoglobin. Under the proper conditions both cause the formation of methemoglobin In the mouse however methemoglobin is formed only with difficulty and when formed tends to be converted rapidly to oxyhemoglobin. Hence in mice only potent drugs like nitrite cause enough methemoglobin to produce cyanosis. Sulfanilamide however, differs from nitrite in also being able to catalyse the formation of sulfhemoglobin when sufficient labile sulfur is available

All groups of mice given sodium nitrite developed an anemia of about the same order of magnitude as mice on sulfanilamide The average loss of hemoglobin varied from 2.3 gram per cent for mice fed one per cent nitrite to 3.2 per cent for mice fed four per cent. The percentage of reticulocytes also increased average changes amounting to about fifteen per cent. Also of interest was the appearance of Heinz bodies in most of the mature erythrocytes of animals on nitrite

Influence of sulfur diets on sulfanilamide cyanosis in chickens

To investigate further the relation between methemoglobin and sulfhemoglobin, we made observations of the effect of sulfur on the action of sulfanilamide in chickens. A previous study demonstrated conclusively that sulfanilamide administered to chickens produced cyanosis characterized by methemoglobinemia (4) The diet of the birds in that study consisted almost entirely of mixed grain, and was low in sulfur It seemed important to determine whether a diet which produced sulfhemoglobin in mice might not

also produce this same abnormal pigment in chickens Accordingly three chickens were fed a diet of Purina Dog Chow containing 2 per cent sulfanilamide In three to five days all the birds became cyanotic Spectroscopic examination of the blood showed only a band of methemoglobin, as reported previously, and there was no indication of sulfhemoglobin In other trials in which 0 5 per cent sulfur and 2 per cent sulfanilamide were added to the diet of chickens, cyanosis was also associated only with methemoglobin It was clear therefore that there was a marked difference in the response of chickens and mice to sulfanilamide, the former responding predominantly by the formation of methemoglobin, the latter by the formation of sulfhemoglobin This interesting species difference deserves further study, as it might throw light on the reason some patients on sulfanilamide form sulfhemoglobin instead of the usual methemoglobin It is possible that since chickens require larger quantities of sulfur for normal metabolism, this requirement is satisfied before enough accumulates for utilization in the formation of sulfhemoglobin

DISCUSSION

The results in this report demonstrate clearly the relation between ingestion of sulfur and the production of sulfhemoglobin by sulfanilamide In general, it appears that sulfides, or compounds which are readily converted to sulfides, are the most effective in the formation of sulfhemoglobin In compounds like magnesium sulfate or thiourea the sulfur is so firmly bound that it can not be used for this reaction

The reactions leading to the formation of sulfhemoglobin remain obscure. However, from the fact that administration of sulfides alone leads to a small amount of inactive hemoglobin, it would seem that sulfanilamide merely acts as a catalyst On the other hand, the production of methemoglobin appears to be a true oxidation which can be produced by a variety of agents

Although no relation has been demonstrated between the amount of inactive hemoglobin formed and the degree of anemia produced, this does not prove that there is no relation between these two toxic reactions It merely shows that formation of sulfhemoglobin *per se* is not necessary to the destruction of red cells Other results, which will be published later, show that when different derivatives of sulfanilamide are compared for their ability to produce blood changes, those drugs producing the most marked anemia also produce the greatest increase in inactive hemoglobin On the other hand, compounds causing little destruction of red cells also produce only a small amount of inactive pigment

CONCLUSIONS

1 The sulfhemoglobinemia of sulfanilamide in mice was found to be dependent on the sulfur content of the diet Of seven sulfur compounds

tested, thiosulfate, flowers of sulfur, and sulfurated potash were the most effective. Magnesium sulfate and thiourea were ineffective.

2 There was no relation between the degree of sulfhemoglobinemia and destruction of erythrocytes.

3 Mice given sodium nitrite in a high sulfur diet developed cyanosis, which was associated only with methemoglobin, and a moderate degree of anemia.

4. Chickens given sulfanilamide in a high sulfur diet developed cyanosis associated only with methemoglobin.

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TOXICITY OF NAPHTHOQUINONES WITH VITAMIN K ACTIVITY IN MICE

MICHAEL B SHIMKIN

From the National Cancer Institute, National Institute of Health, United States Public Health Service, Bethesda, Maryland

Received for publication July 24, 1940

The discovery that vitamin K_1 (*2*-methyl-*3*-phytyl-*1*,*4*-naphthoquinone) and related naphthoquinones correct plasma prothrombin deficiency in the absence of extensive liver damage found prompt and consistently promising clinical applications The scarcity of published reports on the toxicity of the compounds (1–3) prompted the presentation of the following observations on the toxic effects in mice

ACUTE TOXICITY

Mice of the Swiss (albino) and C_3H (agouti) strains and hybrid white mice, 3 to 4 months old and weighing 25 to 30 grams, were used The animals were maintained on a diet of Purina dog chow and an unlimited supply of water

The naphthoquinones[1], as listed in table 1, were dissolved in sesame oil for subcutaneous and oral administrations The amount of the solvent was 0 3 cc or less The toxic dose of *2*-methyl-*1*,*4*-napthoquinone was the same, however, in a series in which the compound was dissolved in 0 1 to 1 0 cc of sesame oil For intravenous injection, the compounds were dissolved in water, 0 5 mgm per cc, and the mice received 0 25 or 0 5 cc in the lateral tail vein At this concentration, the compounds remained in solution at 40°C

The manifestations of the toxicity of all the compounds were practically identical The mice were prostrated within 3 to 5 minutes and died within a half hour after the injection of large doses of *1*,*4*-naphthoquinone and *2*-methyl-*1*,*4*-naphthohydroquinone At lower doses, the onset was later and the symptoms lasted for as long as 72 hours Transitory mild collapse and respiratory embarrassment were noted with 1 6 grams per kilogram of the di-isovalerate subcutaneously and with 0 25 mgm of *2*-methyl-*1*,*4*-naphthoquinone and the hydroquinone intravenously The low toxicity of vitamin

[1] I am indebted to Dr S Ansbacher of the Squibb Institute for Medical Research for the generous supply of the chemicals

K_1 was undoubtedly associated with its slow rate of absorption the compound was still present in the subcutaneous tissue a month after injection

With toxic doses, the mice lay quietly with legs extended and had evident respiratory embarrassment the breathing being extremely slow and labored No convulsions were noted The skin was cyanotic In several instances

TABLE 1

Acute toxicity of naphthoquinones in mice

ROUTE	DOSE	2-METHYL-3-PHYTYL-1,4-NAPHTHOQUINONE		2-METHYL-1,4-NAPHTHOHYDROQUINONE DI-ISOVALERATE		2-METHYL-1,4-NAPHTHOHYDROQUINONE DIPROPIONATE			2-METHYL-1,4-NAPHTHOQUINONE			2-METHYL-1,4-NAPHTHOHYDROQUINONE			1,4-NAPHTHOQUINONE		
		Number	Died	Number	Died	Number	Died	Time	Number	Died	Time	Number	Died	Time	Number	Died	Time
	mgm.							hours			hours			hours			hours
Subcutaneous	200	10	0														
	100	10	0														
	40	10	0	5	0												
	20			10	0	10	10	5–72									
	10			10	0	10	3	44–72	20	20	3–6	10	10	0 25			
	5			10	0	10	1	48	24	23	5–20	10	10	1–8	10	10	0 5–3
	2			5	0	20	0		21	5	16–24	11	8	3–30	10	10	1–6
	1					10	0		36	0		20	0		18	11	4–24
	0 5								20	0		10	0		17	0	
Oral	40	10	0	5	0	10	0										
	20	10	0	10	0	10	0		11	2	1–48						
	10			5	0				10	1	5	10	3	2–3	10	10	1–10
	5								10	0		10	0	0	10	10	2–20
	2								16	0		15	0	0	10	0	6–20
	1														10	0	
Intravenous	0 25	10	0	10	0	10	0		10	0		10*	0		10	1	16
	0 125	10	0			10	0		10	0		10	0		10	0	

* Toxic symptoms present.

the urine became brown in color Death was due to respiratory failure the heart continued to beat for several minutes after the cessation of respiration

At necropsy the liver and the large blood vessels were engorged Upon section of the aorta and other large vessels the blood clotted with extreme rapidity

Histologic sections[2] of the liver, brain, kidneys, spleen, adrenals, lungs, heart, and the genital organs were obtained from 22 mice The findings were the same in all cases, and with all the compounds studied

There was moderate to marked congestion of the organs, particularly of the liver, lungs, and spleen The blood vessels were engorged In 20 mice the tubules of the renal pyramids close to the cortex contained blood This lesion (fig 1) was uniformly focal in distribution It was felt that the renal

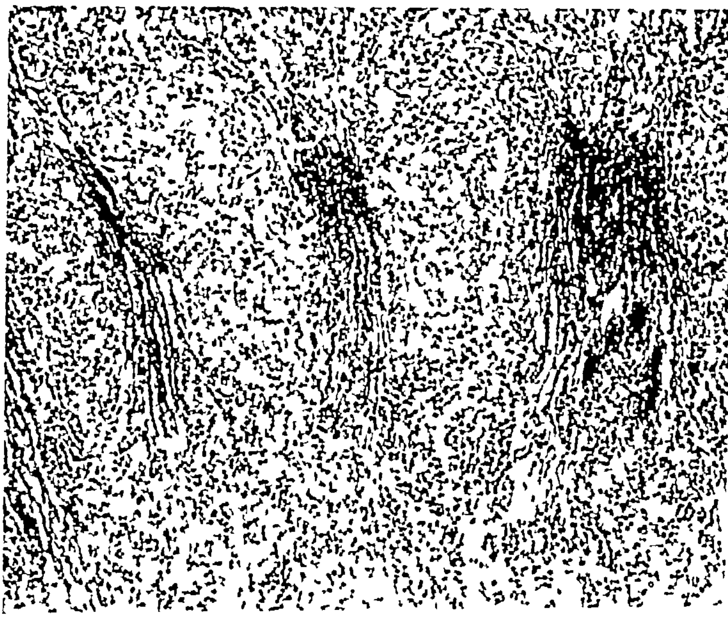

Fig 1 Hemorrhagic Extravasation in Renal Pyramids
Swiss strain mouse 3 hours after subcutaneous injection of 5 mgm of 2-methyl-1,4 naphthoquinone H and E, × 110

lesion was secondary to the marked congestion in the blood vessels In four instances there was some hemorrhagic extravasation into the liver and phagocytosed yellow-brown pigment in the liver, spleen, and kidneys This lesion was not present in four mice which received sublethal doses of the compounds and which were killed at the same time

Six mice which survived the toxic doses of the compounds were killed 3 to 30 days later There was no vascular congestion The most marked residual lesion in the kidney, a large infarct, is shown in figure 2

[2] I am indebted to Dr G Burroughs Mider for review of the histologic material

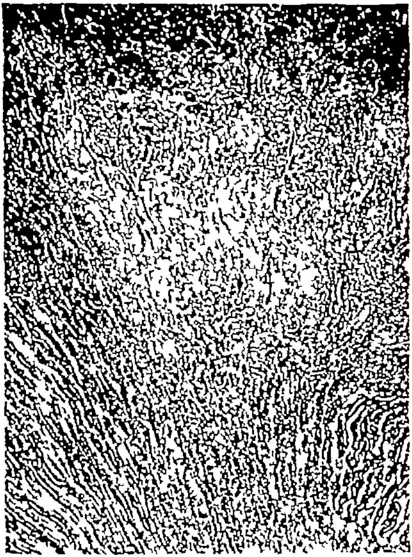

FIG 2 INFARCT OF THE KIDNEY
Swiss strain mouse, 3 days after subcutaneous injection of 20 mgm of 2-methyl 1 4 napthohydroquinone dipropionate H and E, × 45

CHRONIC TOXICITY

Fifty-four Swiss or C_3H mice received periodic subcutaneous injections of 2-methyl 1 4 naphthoquinone or 2-methyl 1 4-naphthohydroquinone dissolved in 0 1 cc of sesame oil per dose as follows

NUMBER OF MICE	COMPOUND	DOSE	INJECTION INTERVAL	TOTAL DOSE
		mgm.		mgm.
10	2 methyl 1 4 naphthoquinone	0 5 × 8	Every other day	4 0
10	2 methyl 1 4 naphthoquinone	1 0 × 8	Every other day	8 0
14	2-methyl 1 4 naphthoquinone	1 0 × 18	Thrice weekly	18 0
10	2-methyl 1 4-naphthohydroquinone	0 5 × 8	Every third day	4 0
10	2-methyl 1 4-naphthohydroquinone	1 0 × 5	Every third day	5 0

Ulceration at the site of injection developed in about half of the Swiss mice, and in three of the 14 C_3H mice There was little cumulative toxocity of the compounds administered at these intervals, since there were no deaths or toxic symptoms

Under the conditions of the experiment, and as compared with the controls which received sesame oil injections, there was no retardation in the gain of weight of the animals, and no significant drop in the erythrocyte count or hemoglobin

Histologic sections of the liver, kidneys, spleen, adrenals, lungs, heart, brain, and genital organs were studied in 29 mice, killed from 1 to 60 days after the final administration of the compounds No significant or consistent morphologic changes were observed in any organ No vascular congestion or kidney hemorrhages were seen The subcutaneous tissue at the site of injection showed a subacute inflammatory reaction

SUMMARY

The acute and chronic toxicity of six naphthoquinones with vitamin K activity were studied in mice The compounds at high doses are respiratory depressants and produce acute vascular congestion which is sufficient to cause hemorrhagic extravasation in the renal tubules and in the liver

No weight loss, anemia, or morphologic changes were observed in mice which were injected subcutaneously with 4 to 18 mgm of *2*-methyl-*1*,*4*-naphthoquinone during 3 to 6 weeks

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EXPERIMENTAL STUDY OF TOLERANCE TO SULFANILAMIDE IN THE ALBINO RAT

ABRAHAM D KREMS ARTHUR W MARTIN AND JAMES M. DILLE

From the Department of Pharmacology College of Pharmacy and the Department of Zoology and Physiology University of Washington Seattle Wash.

Received for publication October 1 1940

While sulfanilamide has been extensively investigated from the standpoint of its action on pathogenic organisms the effect on the host, when it is administered repeatedly deserves further consideration Barlow (1) administered 0.25 grams per kilogram daily to rats for 9 weeks without observing any deleterious effects. Marshall, Cutting and Emerson (2) fed sulfanilamide to young rats for 70 days they found no significant effect on the growth rate and observed no pathological lesions Molitor and Robinson (3) administered 0 5 and 1 0 gram of sulfanilamide per kilogram to rats for 120 days without effect even on the growth rate Two grams per kilogram per day produced rapid loss of weight and death between the 19th and 90th day They found that after 10 days of daily administration the toxicity of sulfanilamide was increased

We have investigated the effect of repeated administration of sulfanilamide on *a* the susceptibility as measured by the response to a median lethal dose, *b* the oxygen consumption of excised liver tissue and *c*, the toxicity of sulfanilamide in vitro to liver tissue

EXPERIMENTAL

The change in susceptibility after repeated administration of sulfanilamide

Preliminary experiments showed that the intraperitoneal dose required to kill 50 per cent of a group of 4 month old male rats (the Median Lethal Dose) was approximately 2.5 grams For all lethal dose determinations the sulfanilamide was administered as a 75 mgm. per cubic centimeter suspension in a 6 per cent acacia solution

Two groups of 4 month old rats were used. The experimental group received 0 4 grams of sulfanilamide per kilogram twice a day for 14 days, given intraperitoneally in 0.8 per cent aqueous solution. The total volume of solution injected amounted to about 10 cc The control group received an equivalent volume of 0 43 per cent sodium chloride solution which is isoosmotic to the sulfanilamide solution. Twenty four hours after the last injection the rats were given a single dose of 2 5 grams of sulfanilamide per

kilogram intraperitoneally The percentage of deaths in the groups is shown in table 1 The dose of 2 5 grams per kilogram which our first study showed to be approximately the L D 50 is now seen to the nearer the L D 60 This, however, does not affect the principle of the procedure The results show that there is a statistically valid (4) decrease in the percentage mortality from a dose of 2 5 grams per kilogram in the groups which had received previous treatment with sulfanilamide This indicates an acquired tolerance In addition, an interesting sex difference is shown A far greater percentage of the females died in both the control and treated groups

An approximate determination of the Median Lethal Dose was made on twenty four male rats one month old and found to be 1 8 grams per kilogram

TABLE 1

The response of groups of rats to a dose of 2 5 grams per kilogram of sulfanilamide administered intraperitoneally

Treated rats received 0 4 gram of sulfanilamide per kilogram twice per day Untreated rats received equivalent volumes of sodium chloride solution

CLASS	NUMBER OF ANIMALS	NUMBER DYING	PERCENTAGE DYING	PERCENTAGE DIFFERENCE	σ D PER CENT	NUMBER OF STANDARD ERRORS IN THE DIFFERENCE	ODDS AGAINST CHANCE
			Male				
Control	44	25	59 1	21 6	9 3	2 32	45 to 1
Treated	80	30	37 5				
			Female				
Control	46	44	95 7	13 8	6 3	2 27	40 to 1
Treated	62	54	81 8				

This confirms the finding of Molitor and Robinson (3) that young rats are more susceptible to sulfanilamide than older rats

The change in oxygen consumption of excised liver tissue after repeated administration of sulfanilamide

Four-month old male albino rats in groups of 8 to 12 were used Animals of the experimental groups received 0 4 grams of sulfanilamide per kilogram twice daily One group was treated for two weeks, another for four weeks and a third for six weeks The control group received saline injections twice daily for 2 weeks Twenty-four hours after the last injection each animal was killed by a blow on the head and the liver was immediately excised and sectioned The rate of oxygen consumption was determined on six sections of tissue run simultaneously in simple respirometers The medium employed was mammalian Ringer's solution to which was added glucose to 0 1 per cent,

it was buffered to pH 7.2 with 0 15 molar phosphate buffer In the present paper tissue respiration (QO_2) is expressed in cubic centimeters of oxygen absorbed per gram wet weight per hour at 38°C and 760 mm. Hg atmospheric pressure There is ordinarily some variation (usually about 10 per cent) between QO_2 of various sections taken from the same organ. We have considered the mean QO_2 of all tissue sections used in the same manner as the value for the particular animal

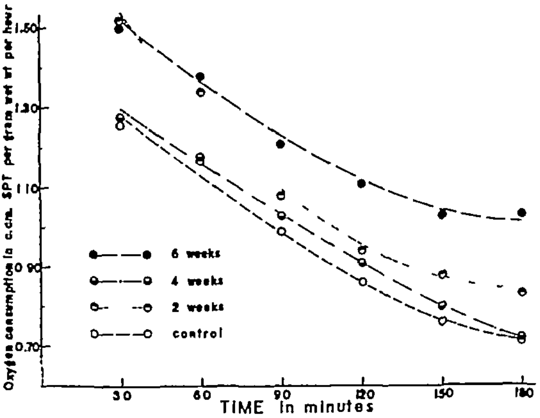

FIG 1 THE RELATIONSHIP BETWEEN THE QO_2 OF EXCISED LIVER TISSUE AND THE DURATION OF TREATMENT WITH SULFANILAMIDE

The experimental groups received 0 4 grams of sulfanilamide per kilogram twice daily for the periods indicated. Each point on the curve is a mean of the values of eight animals.

For brevity all the data gathered (5) are summed up in figure 1 in which we have shown the relationship between QO_2 values and the duration of sulfanilamide treatment Each point on the curve represents the arithmetic mean of at least eight animals It can be seen that there is the usual diminution in the rate of oxygen consumption with the lapse of time after excision which is approximately linear in the control animals The experimental group which had received sulfanilamide twice daily for two weeks shows a significant elevation of the QO_2 only during the first hour This may perhaps be attributed to an oxygen debt in the tissues of the animal The 4 week

experimental group shows no significant deviation from the control group The six week group, however, shows a significant elevation at every point measured The significance of this deviation has been tested with one of Fisher's methods for dealing with small samples (6) and results in probabilities of the order of 1 in 100 that the elevation could have occurred by chance Since the normal trend of the basal metabolic rate in the age range of our animals is downward (7) this effect is not due to increasing age

A possibility exists that temperature changes occurring daily in the animals might account for these results We therefore determined the effect of this

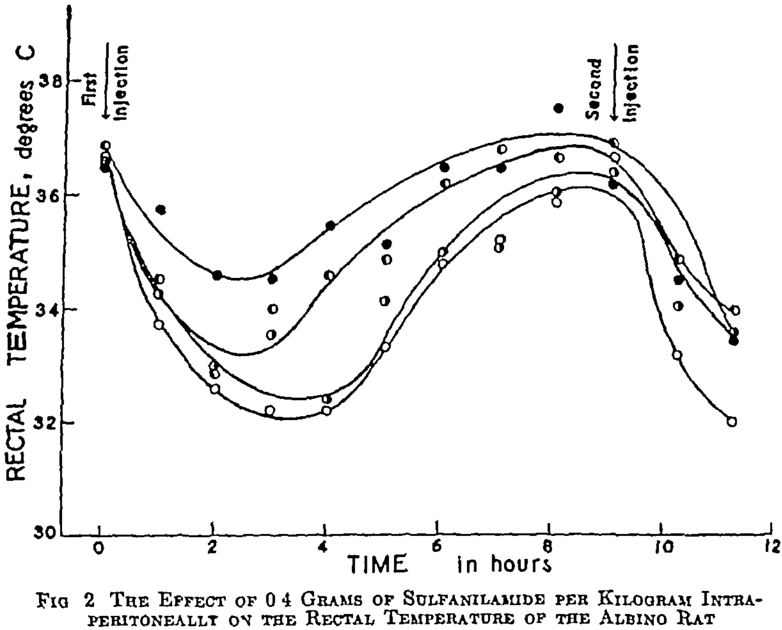

FIG 2 THE EFFECT OF 0 4 GRAMS OF SULFANILAMIDE PER KILOGRAM INTRAPERITONEALLY ON THE RECTAL TEMPERATURE OF THE ALBINO RAT

dose of sulfanilamide on the body temperature A typical experiment is presented in figure 2 It is apparent that sulfanilamide in this dose lowers the body temperature from 5 to 10 per cent as a maximum, well outside of the normal variation for homotherms This effect has been followed for two weeks and occurs throughout that time With two doses daily the rats must have been below normal body temperature for a considerable part of the day We may assume that the decreased body temperature has given rise to an increase in metabolic rate Since the metabolic rate of excised tissue should show a definite relationship to the metabolic rate of the intact animal (8)

this temperature effect may possibly account for the increased tissue respiration.

The change in toxicity of sulfanilamide in vitro to liver tissue of rats after repeated administration of sulfanilamide

The same groups of rats in the above experiments were made use of in another way Following a preliminary determination of the QO_2 of liver slices, 15 mgm of dry sulfanilamide was rinsed in from the sidearm of the respirometer vessels of each of three samples The other three were continued without such addition and, from the normal diminution of their rates

TABLE 2

Percentage decrease in the rate of oxygen consumption of excised liver tissue with and without the addition of sulfanilamide in vitro

Rats which had previously received sulfanilamide were given 0 4 gram per kilogram twice per day Each figure represents the mean of 8 to 12 cases

PERIOD OF TWICE DAILY SULFANILAMIDE ADMINISTRATION	LIVER SLICES—UNTREATED PER CENT DECREASE	LIVER SLICES—TREATED WITH 15 MGM. OF DRY SULFANILAMIDE PER CENT DECREASE	DIFFERENCE DUE TO THE SULFANILAMIDE
First hour			
Control	24 5	43 4	18 9
Two weeks	27 3	50 6	23 3
Four weeks	18 2	41 1	22 9
Six weeks	19 3	37 3	18 0
Second hour			
Control	35 6	59 4	23 8
Two weeks	35 9	71 7	35 8
Four weeks	32 6	58 9	26 3
Six weeks	26 1	49 4	23 3

correction was made to determine the exact effect of the added sulfanilamide The percentage decrease from the first hour QO_2 was calculated and the values given in table 2 are means of at least 8 rats No evidence could be demonstrated by this means of tolerance to the daily injections of sulfanilamide.

DISCUSSION

A tolerance to sulfanilamide can be produced in rats by administering the drug for a period of time We have shown that if a group of rats is treated with twice daily administrations of sulfanilamide for two weeks the percentage of that group dying from a Median Lethal Dose is less than that of a control group This is in contrast to the increased toxicity found by Molitor

and Robinson (3) We think this difference is due to different routes of administration Perhaps in their work, when the lethal dose was determined some sulfanilamide remained in the gastro-intestinal tract, or in the body, from the last of the daily doses, or perhaps the final dose was more rapidly absorbed, as a result of the previous oral administration, leading to an apparent increased susceptibility

Since it has been suggested that respiratory mechanisms are intimately involved in the action of sulfanilamide in the body (9) it was of considerable interest to see if any change could be detected in the QO_2 of a tissue such as liver even though it has been reported (3) that there is no change in the basal metabolic rate of the intact animal The observation that the QO_2 is significantly elevated in the first hour after death in animals treated for two weeks with sulfanilamide is interesting but difficult to interpret We have entertained the idea that this may be a reflection of the general state of the animal in which the blood picture should be considerably upset (10) and so may be of the nature of recovery from an oxygen debt

The elevation of the QO_2 at the six week period is of more theoretical consequence The simplest interpretation would attribute this rise simply to a physiological adjustment of the mechanisms of heat production due to the reduced body temperature Since this would be an interesting point physiologically we have undertaken to answer this question later

We have thought of the possibility that tolerance might be demonstrated by changes in cellular respiration The likelihood still remains that for other drugs a simple and sensitive determination of tolerance could be made in this way In the case of sulfanilamide, although we have clearly shown by in vivo studies that tolerance was developed, there was no decrease in susceptibility of the tissue respiration mechanisms to this drug In fact at the two-week interval, when tolerance had developed, the liver tissue gives some indication of slightly increased sensitivity to added sulfanilamide

CONCLUSIONS

Twice daily intraperitoneal administration of 0 4 grams of sulfanilamide per kilogram to rats produces a decrease in their susceptibility to a dose of 2 5 grams per kilogram intraperitoneally

Female rats are more susceptible than males to sulfanilamide The L D 50 for 1 month old rats is much less than that for 4 month old rats

Liver tissue from rats which had received sulfanilamide twice daily for six weeks shows a slight elevation in QO_2 We have found that the body temperature was lowered by these doses and attribute the increased QO_2 to a physiological readjustment

Susceptibility to sulfanilamide, as measured by the QO_2 of excised liver tissue, is not decreased by daily administration of sulfanilamide

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EFFECTS OF CONTINUED CADMIUM FEEDING[1]

ROBERT H WILSON, FLOYD DEEDS AND ALVIN J COX, JR.

From the Bureau of Agricultural Chemistry and Engineering, U S Department of Agriculture, at the Department of Pharmacology, and from the Department of Pathology, Stanford University School of Medicine, San Francisco, California

Received for publication October 7, 1940

A survey of the literature dealing with the toxicity of cadmium shows that there exists considerable information on the acute toxicity of this metal, but that studies of chronic toxicity are practically limited to the reports by Alsberg and associates (1, 2), and Prodan (3) An adequate review of the literature has been published by Prodan (4)

In addition to the observations of these workers, there are reports which indicate that cadmium may be a general protoplasmic poison Gerber (5) showed that cadmium retarded the action of various rennins, and that the activity of amylases (6) might be either retarded or completely checked, depending upon the cadmium concentration A 50 per cent inhibition of the proteolytic action of papain by a concentration of 1 3 $\times$ 10^{-5} mols of cadmium per liter has been demonstrated by Krebs (7) An inhibitory action of cadmium on catalase and peroxidase activity has been shown by Zlataroff (8), and a distinct retardation of the inversion of sucrose and the transformation of acetic acid by dried yeast in the presence of low concentrations of cadmium, by Kostytschew and Medwedew (9) According to Athanasiu and Langlois (10), cadmium sulfate, in a concentration of 0 18 to 0 2 parts per 1000, completely inhibited lactic acid fermentation Kochmann and Grouven (11) succeeded in killing protozoa with 1 300,000 cadmium chloride Mathews (12) reported that cadmium was more toxic to the eggs of *Fundulus heteroclitus* than was zinc, the concentration of the least toxic dose of cadmium being N/12,500

In perfusing cadmium sulfate through isolated hearts of frogs and turtles, Athanasiu and Langlois (10) demonstrated a decrease in both the rate and force of the heart beat Salant and Connet (13) perfused frog hearts and showed that a concentration of 1 1,000,000 cadmium acetate in Ringer solution caused a slight diminution in frequency and a marked decrease in force of the beat after perfusion for 2 minutes In another experiment the same concentration of cadmium caused complete arrest of the heart in 1 5 minutes Hartwich (14) perfused frog kidneys with cadmium chloride solutions and produced a diuresis which paralleled the velocity of the perfusion flow Athanasiu and Langlois (10) studied the action of cadmium on blood of dogs *in vitro* and *in vivo*, and reported marked hemolysis, formation of hematin and an increase in free serum globulin

Schwartze and Alsberg (1) conducted chronic feeding experiments with cadmium on 17 cats for different periods up to 64 weeks The data on six cats were discarded because quantities of cadmium of 250 to 1,000 parts per million parts of food made regular

[1] Food Research Unit Contribution No 510

feedings impossible In these animals, emesis and refusal of food were frequent and loss of weight occurred. The remaining 11 cats were given 100 grams of meat daily for five days of the week, and 150 grams on Saturday with the same absolute amount of cadmium as on each of the other 5 days The cats receiving 200 parts or less of cadmium per million parts of food either maintained or increased their weight, although there was occasional emesis or refusal of food There was no evidence of a systemic cumulative action. Analytical data showed that cadmium was stored mainly in the liver and kidney and sometimes in the spleen When large doses were given during long periods absorption was more rapid than excretion Elimination of stored cadmium took place through the kidneys.

Johns, Finks, and Alsberg (2) studied the influence of ingested cadmium chloride on the growth of rats. They reported that a diet containing 250 or more parts of cadmium per million parts of diet permitted little or no growth and caused early death. When the diet contained 125 parts per million of cadmium, the initial growth was normal but male rats died within 50 days, whereas three of the five females lived much longer When the concentration of cadmium was lowered to 62.5 parts per million, the food intake and rate of growth remained normal The food intake increased as the concentration of cadmium in the diet decreased but whether diminished food intake was the sole cause of lowered growth rates was not determined. They observed no cumulative action when the daily cadmium intake was 0.56 mgm. per rat.

In view of these general observations on a variety of toxic actions by cadmium, it might be expected that continued feeding of cadmium-containing diets would produce anemia, loss of appetite diminished food intake and retardation of growth. There also might be criteria of chronic toxicity other than those described thus far Because of the inquiries that continue to be received concerning the potential public health hazard from cadmium plated articles it appeared desirable to repeat the feeding experiments with diets containing various concentrations of cadmium. The use in this laboratory of a standardized rat colony in which individual variations in growth rate are minimal, together with the possibility of discovering new criteria of chronic toxicity encouraged the further investigation of this metal.

PROCEDURE

Standard male albino rats weighing 50 ($\pm$5) grams were placed on diets containing 0, 0 0031 0 0062, 0 0125, 0 025 and 0 05 per cent of cadmium as cadmium chloride. The basic diet (15) was that ordinarily used in this laboratory This permits good growth, good reproduction and long life. All animals were weighed and their food intakes determined at weekly intervals, for a period of 100 days. During this time they were observed carefully for any gross evidences of toxicity and the blood was examined to detect possible alterations After noting bleaching of the incisor teeth in the experimental animals special procedures were designed to study this symptom of toxicity These are described in detail in the section dealing with the teeth. At the end of the experimental period all animals were killed and autopsied according to the procedure previously described (16) the

general appearance of the organs noted, the organs weighed and saved for histological examination

Growth and food intake

The data on growth rates are presented as curves in figure 1 The retardation in growth became more pronounced with increasing concentrations

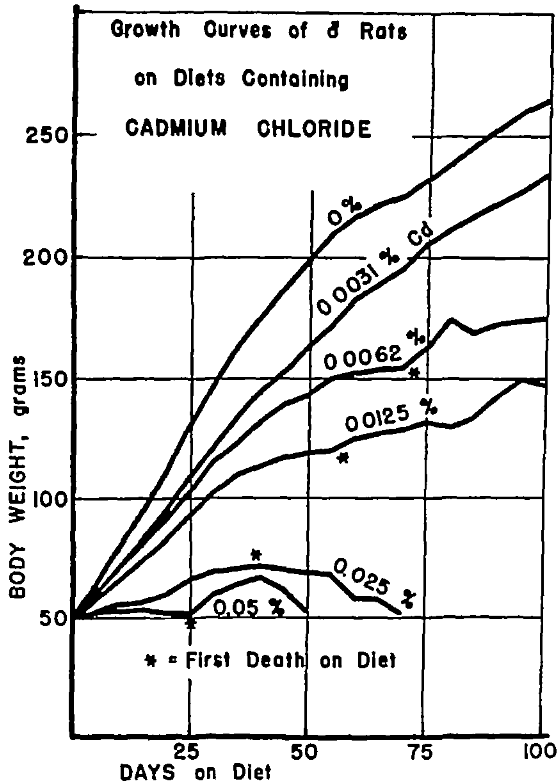

FIG 1 DECREASED GROWTH RATES DUE TO THE PRESENCE OF CADMIUM IN THE DIET

of cadmium in the diet A stunting of growth was evident on a concentration of 0 0062 per cent cadmium In the absence of data on this and higher concentrations, the slight effect produced by 0 0031 per cent cadmium would be of doubtful significance, but when considered in conjunction with the higher levels, it appears that even this low concentration of cadmium was not without effect on growth rate The food intake decreased progressively as the concentration of cadmium in the diet increased Paired feeding

experiments were not made to determine whether or not the decreased rate of growth was due entirely to diminished food intake or involved a specific toxic action traceable to the cadmium. Such experiments were intentionally omitted because other criteria of toxicity were sufficient to establish a case against cadmium. In figure 1 the time at which the first death occurred on each dosage level has been indicated. There is a definite correlation between the time up to the first death and the concentration of cadmium in the diet.

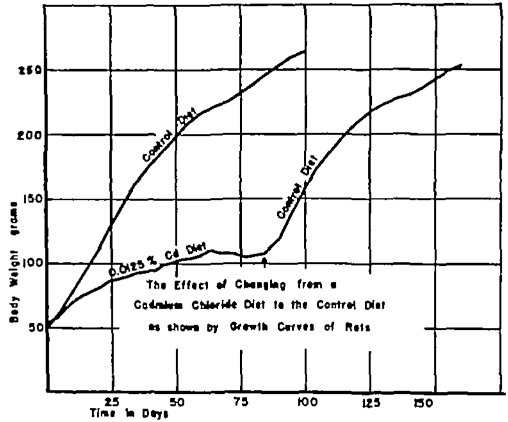

FIG 2 RESUMPTION OF NORMAL GROWTH RATE ON RETURN TO CONTROL DIET

Since 0.0125 per cent cadmium in the diet had a marked effect on growth rate, this dosage level was selected to determine whether or not the stunting of growth was a permanent injury. Rats which had been on a diet containing 0.0125 per cent of cadmium for 84 days were returned to the control diet. The curves in figure 2 show that the rats rapidly attained a normal rate of growth, indicating that so far as this criterion of toxicity is concerned no permanent injury resulted from ingestion of the cadmium.

Blood

Continued feeding of cadmium, even on a dosage level as low as 0.0031 per cent, produced anemia of sufficient severity to cause a distinct decrease

in color of the normally pink eyes of the albino rat When the concentration of the cadmium in the diet was 0 0062 per cent or more, hemoglobin readings dropped from a control value of about 16 5 to 3 or 4 grams per 100 cc of blood within 2 to 3 months, and in one case to 2 6 grams This marked anemia was apparent in several groups of animals, and was accompanied by a lower, though not proportionally lower, number of red cells Anisocytosis and poikilocytosis were present When the rats were again given the control diet in place of the poisoned diet, they not only exhibited a remarkable increase in growth rate, as mentioned previously, but the anemia disappeared within 3 or 4 weeks, judging by the color of the eyes

The final group of rats, eating the 0 0125 per cent diet, was used for the sole purpose of following the blood picture, and was the only group that did not have as severe an anemia The lowest recorded values in this group, in animals kept on this diet for 3 to 7 months, were 6 0 and 5 2 per cent hemoglobin Growth curves were not obtained for these animals, but their general appearance indicated that they were less severely poisoned than previous groups on the same diet No reason for the difference in behavior was apparent During this period between the third and seventh months, the number of erythrocytes ranged from 6,750,000 to 10,050,000 per cu mm as compared to control values of around 8,000,000 to 10,000,000, the cell counts tended to be lower in those animals with the lower hemoglobin values The number of reticulocytes gradually increased from a normal value of 1 to 3 per cent until they accounted for 6 to 8 per cent of the red cells The white cell count remained normal, as did the differential white cell count, except for a suggestion of an increase in number of eosinophils towards the close of the experiment The bone marrow from the poisoned animals was very hyperplastic as compared with the marrow of normal animals In histological sections there was almost complete absence of fat cells, and myeloid cells were approximately twice as numerous as in sections from control animals A study of marrow smears was not made, but no significant qualitative abnormalities were noted in the tissue sections

Effects on teeth

The most surprising and most delicate criterion of chronic cadmium poisoning in the albino rat was found to be the production of bleached incisors similar to, if not identical with, the bleaching produced by fluorine Special feeding experiments designed to study this phenomenon demonstrated that an unmistakable degree of bleaching of albino rat incisors was produced by diets containing 0 0016 per cent of added cadmium in the form of cadmium chloride On this dosage level the rats grew normally and showed no evidence of anemia, or cardiac hypertrophy such as described below

This unexpected and striking observation raises several questions Is the enamel defect produced by cadmium the same as that caused by fluorine?

Does cadmium *per se* produce the effect? Does cadmium sensitize or predispose the animal to the action of the subminimal amounts of fluorine unavoidably present in the diet? In other words, are the effects of cadmium and fluorine synergistic or additive? The following three sets of experiments were made to obtain data relative to these questions

Early in these studies and in those reported elsewhere (17, 18) on the synergistic action of thyroid on fluorine toxicity we found that a graphic method of recording incisor tooth color was a great convenience. When the incisors of the young rat first erupt they are completely white. The anterior enamel surface of the incisors soon acquires a light brownish tinge, which rapidly darkens until it nearly reaches the depth of color seen in the incisors of the normal adult rat (17) The final increase in coloration is a rather slow process For graphic representation of these color changes the degree of coloration was plotted against time. Complete bleaching was represented by +++, and '−' indicated the brown color seen on the tooth of the normal adult albino rat while "++," "+, and '± corresponded to intermediate degrees of bleaching This arbitrary evaluation of the degree of bleaching permitted the observer to duplicate closely his estimation of the degree of bleaching even when not knowing which animal was being examined and the estimation of one observer agreed well with that of another For purposes of plotting, the designations assigned to the individual animals in each group were averaged

In the first experiment four groups of 4 to 6 rats each were used The first group received the usual untreated control diet The second group received the same diet plus 0 0014 per cent, or 14 parts per million, of fluorine as sodium fluoride The third received a control diet containing 0 0031 per cent of cadmium as cadmium chloride The fourth group was fed control diet with both 0 0014 per cent fluorine and 0 0031 per cent cadmium The effects on the color of the incisor teeth are shown graphically in figure 3

It is seen that the control rats developed normally colored teeth in the usual manner, but that the group receiving 0 0014 per cent of added fluorine retained a distinctly noticeable degree of bleaching This observation confirms previous reports (19) from this laboratory The control diet plus cadmium produced a decidedly greater degree of bleaching and the combination of fluorine and cadmium an even greater bleaching action The four groups considered as a whole do not answer the question as to whether or not cadmium is effective *per se*, the action with fluorine being additive, or whether it acts by sensitizing the animal to the small amounts of fluorine native to the basic diet

The most direct method for answering this question would be to compare a control group of animals on a basic diet known to be free of fluorine with a group of rats on the same fluorine-free diet but containing cadmium. If the incisors were bleached cadmium would be the causative agent if there were

no bleaching, then, in the previous experiment, the cadmium action must have been a sensitization to fluorine Unfortunately the preparation of a fluorine-free diet has not yet been accomplished The nearest approach to this was probably made by Sharpless and McCollum (20) Circumstances precluded the preparation of such a low-fluorine diet in our investigation However, it seemed reasonable to assume that the bone ash used in the preparation of the basic diet was the principal source of the fluorine The above experiment was therefore repeated with the following modifications The bone ash was omitted from the basic diet, but approximately the same con-

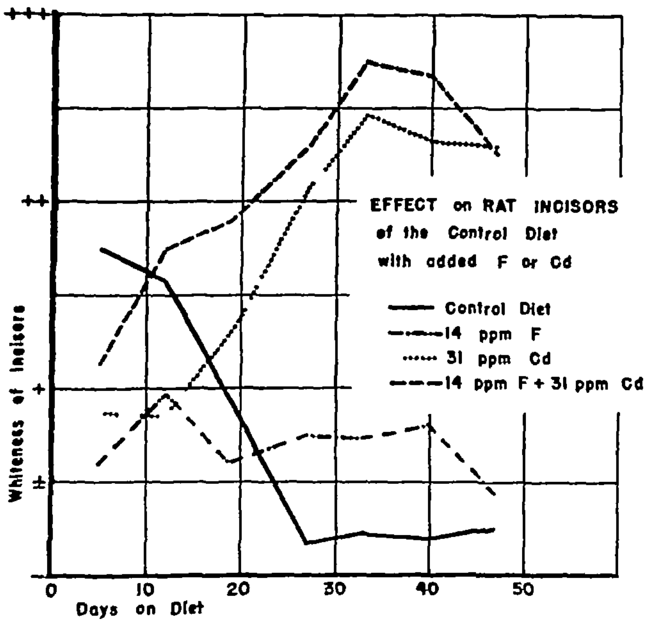

FIG 3 BLEACHING OF RAT INCISORS BY FLUORINE AND CADMIUM

centrations of calcium, magnesium, and phosphorus were attained by the addition of appropriate pure chemicals Three groups of 3 rats each were used The first group received the supposedly low-fluorine basic diet The second group received the same diet plus 0 0062 per cent cadmium The third group received the basic diet plus 0 0062 per cent cadmium and 0 0014 per cent fluorine The data of this second experiment are shown graphically in figure 4

The curve for the control group shown in figure 4 drops more abruptly toward the base line and remains closer to the base than does the corresponding curve in figure 3 This means that the control rats developed a deeper

color of the incisor teeth and did so more rapidly in this experiment than in the former indicating a lower native fluorine content in the basic diet The other two curves of figure 4 show that the teeth started to color but in about 14 days a bleaching effect became evident and proceeded rapidly thereafter In view of the low native fluorine content of this diet these results suggest but do not prove conclusively, that cadmium *per se* can cause bleaching and that the action is not due to a sensitisation of the rats to fluorine present in the diet Two experiments similar to those shown graphically in figures 3 and 4 were performed using a cadmium concentration of 0 0016 per cent. The results obtained were substantially the same as with the higher concentrations,

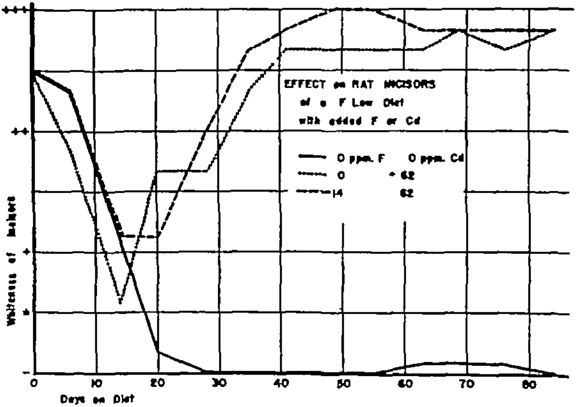

FIG 4. BLEACHING OF RAT INCISORS BY FLUORINE AND CADMIUM

but did not lend themselves to graphic representation because observations were too infrequent in the earlier stages of the experiment.

A third and final attempt to obtain data on the points in question was made, using a different type of diet of low fluorine content. The basic diet employed was that used by MacKay and Callaway (21) This diet contained 25 per cent casein 40 per cent cornstarch, 15 per cent butter fat 10 per cent lard 5 per cent brewer's yeast, and 5 per cent salt mixture In preparing this diet, the Osborne-Mendel salt mixture was used omitting the sodium fluoride Since the other constituents could contribute only small quantities of fluorine the resulting diet presumably contained little native fluorine.

The low native fluorine content of the diet was indicated by the rapid and complete coloration of the control rat incisors, and by observations made during the investigations on the synergistic action of thyroid on fluorine toxicity (17) It was noted in these studies that the basic diet (15) usually employed permitted the development of incisor teeth with normal color at a fairly rapid rate, the native fluorine content being too low to cause a bleaching action, but that after the addition of powdered thyroid to the diet the fluorine present did cause sustained bleaching However, when thyroid was added to the MacKay and Callaway diet, bleaching was only temporary, indicating a much lower native fluorine content

A control group of rats and two experimental groups were placed on this diet exactly as in the preceding experiment The data for this experiment are not given graphically, for the results were nearly identical with those shown in figure 4

There remain to be discussed two other observations which suggest that cadmium itself is effective in producing bleached incisors and that the action is independent of that of fluorine The manner of bleaching by fluorine is fairly uniform The more rapidly growing lower incisors bleach first, and the bleaching of these is quite advanced before an effect is noticed on the more slowly growing upper incisors The lower incisors usually show typical brown and white striations, although, when young rats are placed on a high fluorine intake, complete bleaching may occur so rapidly that the intermediate stage of striation is not observed The bleaching observed in rats receiving cadmium deviated somewhat from the above The upper incisors usually became completely bleached before the lower incisors lost all color The rapidity of the bleaching produced by cadmium was greater than that produced by fluorine, and striations have never been observed on the incisors of rats receiving cadmium This last observation may be due to the rapidity of bleaching

The other interesting observation suggesting that cadmium is effective *per se*, is concerned with the action of cadmium upon the enzyme bone phosphatase *in vitro* Others have found an inhibition of certain enzymatic actions when cadmium was present It has previously been demonstrated in this laboratory that fluorine exerts a toxic action on the activity of bone phosphatase (22), both *in vitro* and *in vivo*, and it has been suggested that this action is important in bone and tooth development Accordingly, observations were made of the effect of various concentrations of cadmium chloride on the ability of an active bone phosphatase preparation to liberate phosphate ion from the substrate sodium glycerophosphate The method was that of Jenner and Kay (23) The experiment was repeated three times, using cadmium chloride concentrations of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} N, and the mean values for phosphatase activities were 8, 10, 16, 66, 70, and 89 per cent, respectively, of the activity in the absence of cadmium

Since a concentration of 10^{-1} N cadmium chloride was required to produce a perceptible decrease in color development in a phosphate standard, the inhibition was on the phosphatase activity and not on the color development

Effects on organ weights

At the time of autopsy, weights were obtained of the adrenals, kidneys, heart, testes, liver and spleen of each animal, in the same way as described previously (16) Organ weights of rats receiving 0 025 and 0 05 per cent cadmium in the diet did not differ markedly from normal because these animals did not survive long enough to permit development of changes Statistically significant increases in organ weights were found for the adrenals, kidneys heart, and possibly the spleen, in rats receiving a dosage of 0 0125 per cent cadmium in the diet for 100 days The most striking weight increments were found in the heart The hearts of the rats receiving 0 0031 per cent cadmium in the diet were normal in weight. All of the rats eating the diet containing 0 0125 per cent cadmium had markedly enlarged hearts the mean weight being almost double that of the appropriate controls The mean weight of the hearts of animals on the 0 0062 per cent diet was greater than that of their controls but this increase was not very significant However, the data showed that the more highly poisoned rats of this group i e., those having the lowest body weights, had larger hearts than their heavier companions. Microscopic examination of the hearts showed definite evidence of hypertrophy of the cardiac muscle in those animals receiving 0 0125 per cent cadmium

Several investigators have reported kidney damage in cases of cadmium poisoning It is known that certain types of kidney damage produce hyper tension which in turn leads to cardiac hypertrophy Such a mechanism was at first considered as responsible for the cardiac enlargement observed in the cadmium-poisoned rats but the hearts were uniformly enlarged and did not show the hypertrophy limited to the left ventricle as would be the case if hypertension were the causative factor In the section dealing with the effects on blood the continued feeding of cadmium was shown to produce a severe anemia. Daniels and coworkers (24, 25) found that in the rat severe anemia resulted in a marked hypertrophy of the heart We therefore suggest that the rats chronically poisoned by ingestion of cadmium developed en larged hearts secondarily as the result of the severe anemia produced by the cadmium Stephens (26) in 1920 reported several human cases of industrial cadmium poisoning His findings were concerned mainly with the liver but he did report evidence of chronic interstitial nephritis and cardiac hyper trophy in one case

The increased weights of kidneys and spleens of the rats receiving 0 0125 per cent cadmium are in harmony with the observations made on the histology of these organs.

Histological findings

Histological examinations were made of sections of tissues stained with hematoxylin-eosin The heart, lung, spleen, kidney, and liver of all rats were examined, and in some cases the testis, pancreas, stomach, intestine, adrenal, and thyroid The animals on the two highest doses of cadmium, i e , 0 05 and 0 025 per cent, showed frequent anatomical changes in the pancreas, liver, kidney, and spleen

The pancreas was not sectioned from the animals on the 0 05 per cent cadmium diet nor from one animal of the 0 025 per cent group, but in three other rats of this group the pancreas showed marked atrophy with complete replacement of many acini by fat tissue There was evidence of variable subacute pancreatitis The islets of Langerhans were still present and were sometimes isolated in regions where all acini had disappeared

The livers from animals receiving 0 05 per cent cadmium showed slight separation of the cells and frequent vacuolation suggesting that the cells contained lipoid droplets One liver showed a number of focal areas of necrosis, mostly near the lobule centers The livers of rats receiving 0 025 per cent cadmium showed no definite recent necrotic foci, but all showed atrophy of cells in the lobule centers, and in one liver there was infiltration of lymphoid cells associated with slight proliferation of cellular fibrous tissue in these regions

The kidneys of all animals on the two highest doses of cadmium showed some swelling and granulation of the epithelium of the convoluted tubules and formation of hyaline and granular casts, a few of which contained desquamated epithelial cells The glomeruli showed no detectable abnormality

The spleens from most animals receiving the two highest doses of cadmium showed slight hyperplasia of the reticulum cells

The hearts of rats in these two groups were normal, suggesting that rats on these high doses of cadmium did not survive long enough to permit development of cardiac hypertrophy

The organs of rats receiving 0 0125 per cent cadmium for 100 days showed similar, though somewhat more marked, changes The heart muscle was definitely hypertrophied, and in three animals it showed slight to moderate irregular subacute myocarditis with foci of cellular fibrous tissue proliferation The spleens of these rats showed somewhat more hyperplasia than those of the animals on the two higher dosage levels Five of the eight rats on this dosage of cadmium had livers in which the lobule centers showed focal areas of disappearance of liver cells and infiltration of lymphocytes with proliferation of cellular fibrous tissue These lesions were more pronounced than those in the larger dose groups, although the livers of three of the eight rats showed no demonstrable lesions Sections of pancreas from each of eight rats showed marked atrophy with scattered lymphocytes in the stroma The kidneys all showed changes of the type previously described Three rats maintained on a diet containing 0 0125 per cent cadmium for 84 days and subsequently returned to the control diet for 103 days showed relatively little anatomical abnormality, although there were a few casts in the kidney tubules The thyroid of one rat appeared unusually *filled with non*-vacuolated colloid and the follicular epithelium showed less evidence of proliferation than usual

Rats receiving half this dosage, or 0 0062 per cent cadmium, showed less marked, but definite, similar changes in the liver, spleen, pancreas, and kidneys Liver lesions were found in only two of the group, and pancreatic lesions in four of eight animals examined Many animals in this group had ulcers of the colon

In the group of rats receiving 0 0031 per cent cadmium in the diet the lesions were even less marked None of the livers showed demonstrable necrosis, though several

showed foci of lymphoid cells The spleens were considered normal in 5 animals. Of 11 animals in which the pancreas was examined, 8 were normal, while three showed slight atrophy or pancreatitis. The thyroids of two rats showed less than the usual evidence of activity

Summarizing the evidence on pathological changes observed in stained tissue sections, it may be stated that the most striking changes were found in the heart, pancreas and liver The latter showed focal necroses with some inflammatory reaction and local fibrous tissue proliferation The pancreas showed marked atrophy and pancreatitis. The hearts were hypertrophied and a few showed slight irregular inflammation and fibrous tissue proliferation. The spleens were hyperplastic, and the kidneys showed slight epithelial swelling and cast formation. The frequency and significance of the ulcers of the colon could not be determined, since in many instances this organ was not sectioned. The changes observed in the various organs were roughly proportional to the amounts of cadmium received by the animals. The relatively slight changes in animals receiving the largest dosages are probably to be explained by the short survival time of the rats The organs of rats receiving the smallest quantities of cadmium were nearly normal although some showed definite anatomical changes.

Atrophy of the pancreas, together with observations of the frequent very soft stools of cadmium-poisoned animals, suggested the possibility of poor digestion and absorption of fat This was briefly studied Three groups of rats (on the control 0 0031 per cent and 0 0062 per cent cadmium diets) had been fed for 100 days, and one group on the 0 0125 per cent diet for 50 days Feces were collected from these 4 groups dried at 80 C and exhaustively extracted with ether in a Soxhlet apparatus On the basis of dry weight, the weight of ether extract increased very slightly with increasing concentrations of cadmium in the diet This experiment was not repeated Smears were made from crushed feces, stained with Sudan IV, and examined microscopically for fat There was no more stained material in the feces of the cadmium-rats than in those of the controls. Apparently there was little or no decrease in fat absorption

DISCUSSION

The effect of cadmium-containing diets on the growth of the white rat, as reported by Johns, Finks and Alsberg (2), has been confirmed by these experiments with only minor differences In our study the cadmium was found to be somewhat more toxic than these authors reported, and their suggestion of a sex difference has not been corroborated

The blood picture as described in the literature is not entirely consistent Prodan (4) in his review stated that in feeding experiments there was usually a decrease in hemoglobin, and an increase when the cadmium was administered through the respiratory system That this was not always the case was shown in his experiments with cats receiving 2 to 100 mg of cadmium daily in the food for 1 to 2 months (3) He found only one of his animals with an abnormal hemoglobin value and this was high. No anemias as severe as we found in rats seem to have been reported It is quite probable

that the cardiac hypertrophy observed in our animals was due entirely to this severe anemia The literature, in general, contains reports of a relative increase in polymorphonuclear leucocytes The white cell count in our rats remained normal, and the differential count was not modified to any significant degree

Cadmium administered through the respiratory tract is said to produce histological changes in the lungs When given by mouth, the lungs have generally been unaffected Most descriptions of the microscopic examination of tissues mention changes in the kidneys variously described as nephritis, or fatty degeneration or infiltration of the tubules The description in this report indicates slight epithelial swelling and cast formation Cadmium is reported to produce fatty livers, and Prodan (3) found occasional areas of necrosis in cats Areas of focal necrosis were the principal hepatic lesions in our rats Few other histological findings have been mentioned It is not always clear whether or not the other tissues were examined Prodan (3) specifically mentioned the pancreas as being normal in at least one case No pancreatic lesion such as that found in our rats seems to have been described previously

Bleaching of the incisor teeth of the rats receiving cadmium was an unexpected finding It is the most delicate criterion of cadmium poisoning thus far observed, marked bleaching occurring with concentrations of cadmium too low to affect the growth or general appearance of rats It appears to be a direct action of the cadmium, although the possibility of stimulation of fluoride action has not been entirely ruled out

SUMMARY AND CONCLUSIONS

Cadmium chloride was administered in the food to albino rats so as to check and extend the observations of previous investigators The following results were obtained

1 Concentrations of 0 0031 per cent or more of cadmium in the diet resulted in decreased rates of growth, the greater effects being obtained with the higher concentrations of cadmium Death occurred progressively earlier with increasing concentrations of cadmium Rats receiving 0 1 per cent cadmium in the diet lived only a few days

2 A severe anemia occurred in those rats eating diets containing as little as 0 0062 per cent cadmium Within 2 or 3 months the hemoglobin concentration had decreased to 3 or 4 grams per 100 cc of blood The number of erythrocytes decreased, but not to a proportionate degree The white cell count and the differential count remained normal The bone marrow from anemic animals was hyperplastic, suggesting that the marrow itself was not injured, which agrees with the observation of an increase in number of reticulocytes in these animals

3 The incisor teeth of the rats were bleached rapidly and markedly, with

an unmistakable degree of bleaching apparent in animals on the diet containing 0 0016 per cent cadmium This action was probably caused directly by the cadmium although an increase in activity of fluoride in the presence of cadmium has not been definitely excluded

4 The more severely poisoned rats had hearts which weighed nearly twice as much as normal. This cardiac hypertrophy was due presumably to the severe anemia Adrenals kidneys, and perhaps the spleen were heavier than normal

5 Histological examination showed hypertrophy of the heart focal necroses with some inflammatory reaction and fibrous tissue proliferation in the liver and marked atrophy and inflammation in the pancreas There were hyperplasia of the spleen and, in the kidneys, slight epithelial swelling and cast formation.

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ON THE RÔLE OF THE LIVER IN THE DETOXIFICATION OF STEROID HORMONES AND ARTIFICIAL ESTROGENS

HANS SELYE

From the Department of Anatomy, McGill University, Montreal, Canada

Received for publication November 19, 1940

Although a good deal is known about the metabolism of hormones and the end products into which they are transformed after performing their actions in the body, we have little definite information concerning the site at which they are inactivated Heavy overdosage with estrogenic compounds may lead to degenerative changes in the liver (1, 2) Furthermore Pincus and Martin (3) recently reviewed the evidence suggesting that the liver may play an important rôle in the inactivation of the estrogens but there is no uniformity of opinion on this subject and even less is known about the site at which other hormones are detoxified The recent discovery that acute overdosage with various steroid hormones (progesterone, desoxycorticosterone, androgens, and natural as well as artificial estrogens) causes deep anesthesia (4, 5) suggested that the site of detoxification of these compounds might be investigated using the same methods which we had previously employed in our studies on the detoxification of other anesthetics We found that the median and left lobes of the liver of mice and rats may easily be removed after placing a single ligature around the hilum of both these lobes which together represent more than two-thirds of the total liver tissue Since the remaining third of the liver rapidly regenerates, tests on such a preparation must be performed soon after the animal recovers from this relatively simple intervention Rodents thus partially hepatectomized show a normal resistance to anesthetics such as ether or magnesium chloride, which are not detoxified in the liver, but they recover very slowly or may actually die after treatment with comparatively small doses of an anesthetic such as tribromethanol (Avertin), which is inactivated in the liver tissue (6) It was therefore, decided to take advantage of the newly discovered anesthetic effect of the steroids to establish whether these are also inactivated in liver tissue

METHODS

Young female albino rats weighing 55 to 76 grams were used for these experiments since previous observations indicated that females are more sensitive than males and that, in either sex, young animals are more sensitive than adults The steroid hormones used were desoxycorticosterone acetate, progesterone, α-estradiol and testos-

terone benzoate. In addition to these stilbestrol (4 4'-dihydroxy-α-β-diethylstilbene) was also tested because it possesses great estrogenic activity although it is not a naturally occurring hormone and does not contain the cyclopentanophenanthrene nucleus characteristic of the steroids All these substances were administered in solutions containing 20 mg of the active compound per cc of peanut oil 5 mg were injected intraperitoneally every 30 minutes until the desired anesthetic effect was obvious The intraperitoneal route of administration had to be chosen for when given otherwise absorption is not rapid enough to produce anesthesia

The partial hepatectomy was performed by the Waelsch and Selye technique (6) Sixteen hours after the intervention all animals of this experimental series were first tested for their resistance to anesthetics which are not detoxified in the liver For this purpose they were first deeply anesthetized with ether and one hour after recovery they were again anesthetized by subcutaneous administration of 0 7 cc of a magnesium chloride solution These preliminary tests confirmed that the intervention of partial hepatectomy does not alter resistance to all anesthetics In the case of narcosis produced by ether and magnesium chloride the time required to produce deep anesthesia

TABLE 1

Influence of partial hepatectomy on resistance to acute overdosage with steroid hormones and stilbestrol

COMPOUND	TOTAL DOSE	INTACT ANIMALS		PARTIALLY HEPATECTOMIZED ANIMALS	
		Anesthesia	Deaths in groups of 6 rats	Anesthesia	Deaths in groups of 6 rats
	mgm.				
Progesterone	5	Light		Deep	4
Desoxycorticosterone acetate	5	Trace		Deep	3
α-Estradiol	40	None		Light	
Testosterone benzoate	40	None		Light	
Stilbestrol	30	None		Deep	5

as well as the time necessary to recover from it was identical in the partially hepatectomized and in the intact animals After completion of these preliminary investigations the animals were used for the main experiment which was started 24 hours after the operation

RESULTS AND DISCUSSION

Our observations are summarized in Table I which is self explanatory It will be seen that all compounds tested have a much more pronounced action in the partially hepatectomized than in the intact control animals. Most probably the reason why estradiol did not produce as deep anesthesia as stilbestrol is that owing to its greater solubility the latter compound is more readily absorbed It seems likely that a delay in absorption is also responsible for the relatively slight effect of the testosterone benzoate in comparison with the much greater potency of the free testosterone described in our earlier publications. We should also like to draw attention to the fact that in the young animals of this series, progesterone proved more active than desoxy corticosterone while in older animals the reverse was often observed

These experiments suggest that the liver plays an important rôle in the detoxification of steroid hormones. The possibility that the resistance of the partially hepatectomized animals was decreased only in a non-specific manner as a result of the intervention can be refuted because our preliminary tests indicated that the ether and magnesium chloride resistance of these animals is in no way affected by the operation. We must conclude, therefore, that these hormones are either inactivated in the liver or else their inactivation outside this organ is dependent upon metabolic conditions regulated by the liver. In view of the fact that several steroids are known to be inactivated by conjugation with other compounds (glucuronic acid, sulphuric acid) and that such conjugations occur mainly in the liver, the former appears to be the more likely theory.

SUMMARY

Experiments in the rat show that the anesthetic action as well as the toxic effect of acute overdosage by the intraperitoneal administration of desoxycorticosterone acetate, progesterone, testosterone benzoate, α-estradiol and stilbestrol is greatly increased in animals in which the major part of the liver has been removed. Since this operation does not increase sensitivity to other anesthetics such as ether and magnesium chloride, but is known to sensitize animals to the action of anesthetics which are detoxified in the liver (e.g. tribromethanol), it appears most probable that the liver is the site at which all the above mentioned compounds are normally detoxified.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Committee on Research in Endocrinology of the United States National Research Council. The author is greatly indebted to Drs. Gregory Stragnell and Erwin Schwenk of the Schering Corporation of Bloomfield, New Jersey for the steroid compounds and the stilbestrol used in these experiments.

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THE DISTRIBUTION OF INJECTED ORGANIC DISELENIDES IN TISSUES OF TUMOR BEARING ANIMALS[1]

SAUL B GUSBERG PAUL ZAMECNIK AND JOSEPH C. AUB

From the Medical Laboratories of the Collis P Huntington Memorial Hospital of Harvard University

Received for publication August 9 1940

The literature of chemotherapy is large in relation to cancer, but small in the fundamental study of substances which will be concentrated in tumors. The review articles of Sugiura and Benedict (1) and, more recently, Shear (2), well illustrate this fact. The obvious importance of ascertaining the fate of injected substances in tumor bearing animals suggests that such studies are necessary correlaries of chemotherapeutic ones. This problem can only be approached with substances which can be estimated quantitatively in great dilution. Selenium compounds appeared interesting because of their relationship to sulfur metabolism physiological oxidations, and growth. We therefore decided to use these compounds for the first of a series of experiments designed to study the ability of various inorganic substances to penetrate into tumors. We believed that organic diselenide compounds, being similar in structure to several physiological substances, were the most suitable form of selenium for metabolic and tumor studies. This type of selenium compound[2] was therefore used for experiments in which we studied the distribution of selenium in the tissues, the penetrability of selenium into animal tumors and the effect of these selenium compounds on the growth of the tumors

I. THE PENETRATION OF SELENIUM INTO TUMORS

Experimental method The various organic selenium compounds to be injected were tested for their toxicity on mice The minimal intravenous dose necessary to kill all animals in each tested group (at least 10 animals for each compound) was then established, and half of this L.D 100 was used for each injection in subsequent observations. This half L.D 100 dose was used since it proved to be a quantity which could be administered repeatedly in daily doses without external evidence of abnormal reaction in the experimental animals. In the animals given diselenide mercury diacetic acid which did react abnormally in the latter days of the injection period, this dosage was thereafter reduced.

Adult male and female Strain A (Bar Harbor) mice were inoculated in the groin with *sarcoma 180* Two weeks after inoculation when the tumors were definitely visible,

[1] This is reprint No 518 of the Cancer Commission of Harvard University

[2] These compounds were synthesized for us by Dr Jesse Greenstein, who at that time was in the Department of Physical Chemistry Harvard Medical School

daily injections of the selenium compounds were begun These injections were given intravenously, in a tail vein, for 15 days, at the end of which time the animals were killed by bleeding and their tissues analyzed for selenium

Analytical method Horn's modification of the codeine sulfate reaction for the detection of selenium (3) was adapted to work with animal tissues and made roughly quantitative by the use of colorimetric comparisons with selenium-sulfuric acid standards While our experiments were in progress, Gortner and Lewis (4) published details of a method which is in principle the same as the one we used, with the added advantage of being a little more accurate (4) To a sulfuric acid and mercuric oxide digest of the

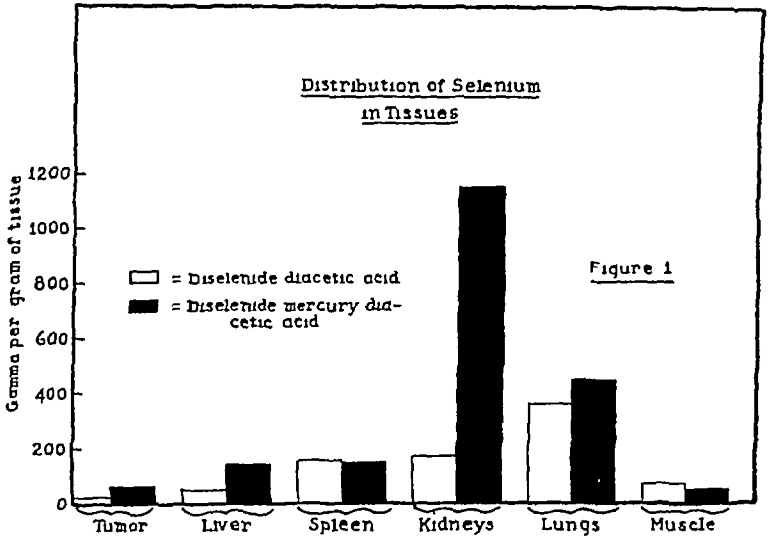

FIG 1 DISTRIBUTION OF SELENIUM IN TISSUES FOLLOWING THE INJECTION OF TWO SELENIUM COMPOUNDS

Eight mice were given 15 daily intravenous injections of 0 5 mgm of diselenide diacetic acid (57 per cent selenium by weight) Three mice were given 15 daily intravenous injections of 0 5 mgm of diselenide mercury diacetic acid (33 per cent selenium by weight), but appeared sluggish at times The five remaining mice in this group were therefore given 0 25 mgm daily for the 15 days, and exhibited no abnormal reactions The results are expressed in gamma (micrograms) of selenium per gram of dried tissue

dried sample a codeine sulfate solution was added Whereas we made colorimetric comparisons one-half hour after development of the blue color, Gortner and Lewis kept the preparations for seven hours in the absence of light, thereby apparently obtaining a greater stability in the blue color used for colorimetric comparison The order of accuracy which we obtained, however, was certainly sufficient for comparison of the selenium content of various tissues The advantages of the colorimetric method over other existing procedures (5, 6) are chiefly simplicity of procedure and speed

Discussion of results

(a) *Penetration into tumor* Figu[illegible] illustra[illegible] results of these experiments It is apparent that in[illegible] [illegible] fraction

of the recovered selenium found in the tumor Furthermore, the concentration in the tumor averaged less than the concentration in muscle—except in the case of diselenide mercury diacetic acid with which there was a slight, though not significant, preponderance in tumor

(b) *Distribution* In all the experiments only a small portion of the injected selenium was found in the tissues, a result which agrees with the work of other investigators (7 8 9) In experiments in which inorganic selenium was injected subcutaneously, Smith *et al.* found that 50 to 80 per cent of the

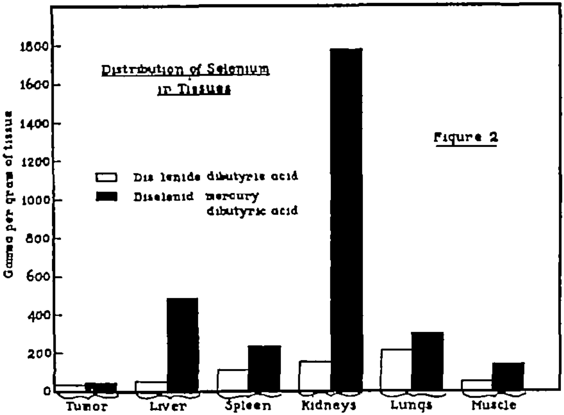

FIG 2. DISTRIBUTION OF SELENIUM IN TISSUES FOLLOWING INJECTION OF DISELENIDE n-DIBUTYRIC ACID AND DISELENIDE MERCURY n DIBUTYRIC ACID

Nine mice were given 15 daily intravenous injections of 1 mgm. of diselenide-n dibutyric acid (48 per cent selenium, by weight) Six mice were given 15 daily intravenous injections of 0.5 mgm. diselenide mercury n-dibutyric acid (30 per cent selenium, by weight)

injected selenium was excreted in the urine, and from traces to 18 per cent in the feces

Our diselenide-diacetic acid (CH_2COOH-Se-Se-CH_2COOH) and diselenide-n-dibutyric acid (C_3H_6COOH-Se-Se-C_3H_6COOH) groups showed the selenium to be most concentrated in the lungs with the kidneys spleen, and liver following in that order This is in accord with the work on inorganic selenium by Dudley (7) who also noted considerable amounts in the liver kidneys and spleen, however he and other investigators (Smith Westfall

and Stohlman (9) and Gortner and Lewis (10)), found lower concentrations in the lungs

The possibility suggests itself that our use of organic selenium compounds, injected intravenously, may have caused greater excretion of the selenium by the pulmonary route We know that the work of Hofmeister (11) and Filippi (12) suggested that selenium could be eliminated by the lungs as a volatile compound, probably methyl selenide Recent workers too have mentioned the garlicky odor of the expired air of selenized animals (Motley, Ellis, and Ellis (13) and others), this has been related to the pulmonary excretion of selenium because of the similar odor of organic selenium compounds However, without confirmatory evidence of this pulmonary excretion of selenium, we do not presume to interpret our own finding of greater concentration in the lungs as an indication of greater excretion there It may be that intravenous injection is an important factor in this distribution to the pulmonary capillary bed, which then has the opportunity to take up a greater percentage than if the selenium had been first offered to the reticulo-endothelial system of the liver

(c) *Effect of change of compound on distribution* In each case the addition of mercury (CH_2COOH-Se-Hg-Se-CH_2COOH and C_3H_6COOH-Se-Hg-Se-C_3H_6COOH) to the organic selenium compound effected a striking change in the distribution of selenium in the tissues (see figs 1 and 2) First, in spite of the fact that less selenium was given in these groups, in almost every case more was found in the tissues It was presumed at first that the combined toxicity of selenium and mercury to the kidneys had probably damaged them enough to cause greater retention of selenium, but examination of histological sections of these kidneys by Doctors Shields Warren and Olive Gates failed to confirm this While Dudley (7) felt he could correlate the amount of selenium in the tissues of his animals with the extent of the pathological changes, we are unable to do so Sections of the kidneys of our animals consistently showed only very slight toxic changes, which did not warrant the conclusion that this was the cause of higher concentrations of selenium in the tissues On the other hand, our lack of histological evidence of renal damage does not exclude this possibility[3]

A second striking effect noted with these mercury compounds was the very great concentration of the selenium in the kidneys, and to a lesser extent in the liver In the light of these chemical and pathological findings, it appears that the addition of *mercury* to the organic selenium compound has changed the distribution of the *selenium* in the body, for it caused a concen-

[3] It is interesting that Westfall and Smith (32) have reported, since the submission of this paper, that selenocystine differs from diselenodiacetic acid in its distribution in fractions obtained by digestion of liver proteins to which these compounds had been added Diselenodiacetic acid behaves in a way similar to the tissue selenium of chronically selenized animals, while selenocystine does not

tration in the kidney which was not found in a similar compound without mercury

II THE EFFECT OF SELENIUM ON TUMOR GROWTH

Franke and his coworkers (14–17) in their intensive investigations of selenium and "alkali disease" demonstrated the definite interference of selenium with development and growth of animals and chick eggs A good part of this diminished growth has been attributed to inanition, but the dis-

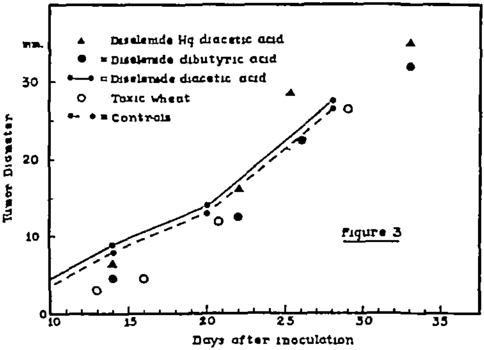

FIG 3 The curves represent the mean of the tumor diameters in the various groups of mice Strain *A* Albino mice were used for the organic selenium compound injections. Strain *C57* mice were used for the toxic wheat feeding

1 Diselenide diacetic acid group 0.5 mgm daily for 15 days in six animals.
2. Diselenide mercury diacetic acid group 0.5 mgm daily for 15 days in five animals.
3 Diselenide-n-dibutyric acid group 0.5 mgm. daily for 15 days in five animals
4. Toxic wheat group North Dakota wheat containing 14 parts per million of selenium, given with milk as the sole diet for 4 weeks to 8 animals.
5 Control groups 29 animals

tinctive pathology seen in animals receiving selenium (18–20) cannot be over looked as a possible factor Inhibiting effects of selenium on the *in-vitro* oxidation of crystalline glutathione (21) on the yeast fermentation of sugars (22) and the oxygen uptake by rat liver (23) have also been demonstrated These latter considerations suggested the study of the effect of selenium on transplanted tumors. Various previous investigations of the effect of selenium on tumor growth have given conflicting results (24–31)

The present experiments on more than 100 mice injected with selenium compounds uniformly failed to demonstrate any selective inhibition of tumor

growth In some of these mice the tumors were smaller than in the control animals, but this was associated with a proportional reduction in total body weight Experiments of Bischoff and Long (33) indicate that simple under-nutrition will produce a similar result, and it appears therefore that these selenium compounds do not have a selective action on sarcoma 180 In addition to injecting organic selenium compounds into 47 mice bearing tumor sarcoma 180, we also injected inorganic selenium compounds (sodium selenide and sodium selenite) in satisfactory experiments on 19 mice without influencing the growth rate of the tumors Nor did 10 mice fed "toxic wheat" (14 p p m selenium[4]) over a period of four weeks show any inhibition of tumor growth Figure 3 illustrates these results

SUMMARY

1 After intravenous injection of selenium into mice as diselenide diacetic acid and diselenide *n*-dibutyric acid, the greatest concentration of selenium was found in the lungs Using similar compounds in which mercury had been added to the organic selenium compound, the greatest concentration was found in the kidneys

2 The addition of mercury to these organic selenium compounds appeared to influence the excretion of selenium, for mice injected with such compounds showed much greater deposition of selenium throughout the tissues

3 It was found that selenium, injected intravenously in the form of diselenide aliphatic compounds or fed in "toxic wheat," did not inhibit the growth of sarcoma 180 in mice Furthermore, tissue analysis of such animals revealed relatively little selenium in the tumors

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The method devised by us utilizes the manometric gas analyzer of Van Slyke and Neill but differs from that of Rinkel and Pijoan in that the enzymatic reaction is carried out in a separate vessel instead of in the reaction chamber of the apparatus. This method lends itself readily to the study of the activity of cholinesterase in any tissue extract or fluid irrespective of whether it is highly colored or turbid The fluid containing cholinesterase is added to a bicarbonate-Ringer solution of pH 7 4 The resulting solution is maintained at a constant temperature, vigorously stirred and kept saturated with a gas mixture of nitrogen and carbon dioxide. An optimal amount of acetylcholine bromide is added as substrate The resulting acetic acid reacts with the bicarbonate liberating carbon dioxide which passes out of the buffered solution The bicarbonate and (in the case of serum and lymph) the proteins present serve to buffer the mixture and to prevent any marked shift in pH. The total content of carbon dioxide is measured by the method of Van Slyke and Neill at three thirteen and twenty three minutes after adding the acetylcholine The index of the activity of cholinesterase is the amount of carbon dioxide in millimoles per liter (mM/l) passing out of the solution within a period of twenty minutes It is calculated from the difference between the carbon dioxide content determined at three and at twenty three minutes It corresponds to the amount of acetic acid split off from acetylcholine within the twenty minute period.

The detailed technique as employed in determining cholinesterase activity in serum and lymph is as follows 1 cc of the serum or lymph is pipetted into a 50-cc. centrifuge tube which is suspended in a water bath kept at 38 C To the serum is added 3 0 cc of a bicarbonate-Ringer solution This is prepared by diluting 10 cc of 10 per cent sodium chloride 0.84 cc. of 10 per cent anhydrous calcium chloride 0 24 cc of 10 per cent potassium chloride and 5 0 cc of 5 per cent sodium bicarbonate to 124 cc. with distilled water and saturating with a gas mixture consisting of 5 per cent carbon dioxide and 95 per cent nitrogen. A flat paddle stirrer is inserted and the solution stirred vigorously care being taken to avoid frothing. A narrow glass tube conducting a stream of the gas mixture is arranged in the vessel so that the nozzle is just above the surface of the solution. A period of 15 minutes is allowed for the sample to reach the bath temperature and to become completely saturated with the gas One hundred and twenty milligrams of acetylcholine bromide dissolved in 0.5 cc. of the bicarbonate-Ringer solution is *added to the solution in the bath. Three minutes are allowed for thorough mixing* The first 1-cc sample is then removed and an analysis for the total content of carbon dioxide is carried out by the procedure of Van Slyke and Neill The reaction is continued and at intervals of exactly ten minutes two more samples of 1 cc each are removed and analyzed for carbon dioxide When the carbon dioxide content of these samples is plotted against time there results a straight-line graph which indicates that the enzyme is working at a uniform rate during the period chosen (see fig 1) The determinations made at three and twenty-three minutes therefore suffice for the calculation of activity

Curve I of figure 1 is a composite of ten different experiments on horse serum. Curve II was obtained by analyzing under the same conditions the bicarbonate-Ringer solution containing acetylcholine bromide but without serum lymph, or any other source of cholinesterase There is some hydrolysis at pH 7 4, which was chosen for these experiments but it is obvious that this error is negligible. Curve III represents the same horse serum as used in Curve I but in this case the activity of the enzyme was inhibited by adding 0 5 microgram of prostigmine methylsulfate for every cubic centimeter of solution, thus making a 1.5×10^{-6} molar solution of prostigmine This concentration of the drug completely inhibits the cholinesterase activity of the horse serum employed.

Accuracy of the method On the basis of numerous analyses on serum we have found repeated determinations to differ by less than 5 per cent Upon addition of known

amounts of acetic acid, the amount of carbon dioxide passing out of the solution agrees within 3 per cent of the calculated value up to 10 mM/l Sera with titers above 10 mM/l must be diluted with bicarbonate-Ringer solution in order to avoid a shift in pH, which must not exceed 0 3 of one pH unit

With this method we examined the activity of cholinesterase in the serum of normal man, horse, dog, and cat (see table 1) Our determinations of the relative activity of

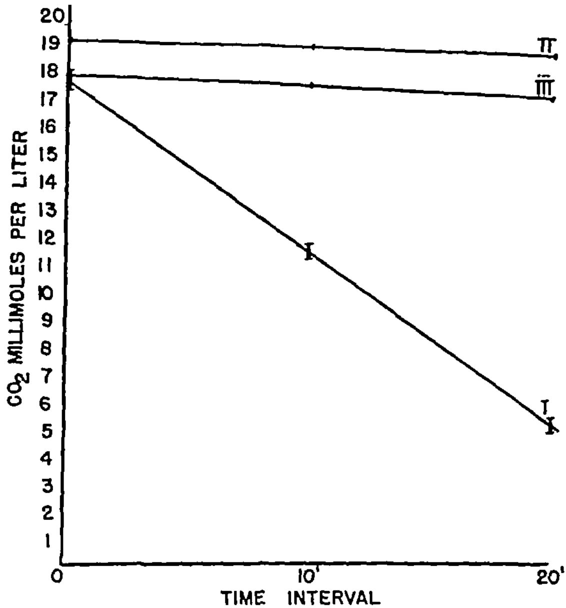

FIG 1 HORSE SERUM CHOLINESTERASE ACTIVITY OF NORMAL SERUM AND OF SERUM TREATED WITH PROSTIGMINE

TABLE 1

Cholinesterase activity of serum, thoracic and cervical lymph before and after prostigmine

SPECIES	NUMBER EXAMINED	RANGE OF ACTIVITY	AVERAGE ACTIVITY
		CO_2 mM/l	*CO_2 mM/l*
Horse	4	10 6–18 6	14 0
Man	10	7 1–17 9	12 3
Dog	18	4 2–11 8	8 9
Cat	5	2 8– 4 1	3 5

the sera of these four species of animals agree with the findings of other investigators (12, 14), although the absolute units differ according to the method used

CHOLINESTERASE ACTIVITY IN LYMPH

The experiments were performed on dogs under anesthesia As a rule the animals breathed spontaneously, but in some experiments artificial respiration became neces-

sary because of pneumothorax. Nembutal was used as a general anesthetic. It was given intraperitoneally or intravenously in a 5 per cent solution and in a dose of 0 033 gram per kilogram body weight. If in the course of the operation or during the experiment the anesthesia became too light, additional doses of Nembutal were administered intravenously (see table 2) As can be seen from a comparison of the serum titers in table 3 (average activity 8 2) with the values for the serum of dogs in table 1 there is no significant difference in the range of activity and in the average activity of cholinesterase of the serum of normal dogs and of dogs under Nembutal We assume therefore that Nembutal in the doses applied in these experiments, is without distinct effect on the activity of the cholinesterase in serum *in vivo* We have not investigated whether this also holds true for lymph.

The lymph was collected from the thoracic duct and from the left cervical duct, which were cannulated before their entry into the left subclavian vein. In order to prevent the lymph from clotting small quantities of heparin[3] were introduced every five to ten minutes into the tip of the cannula. We assume that the quantities of heparin dissolved in the lymph do not influence the activity of cholinesterase as heparin in similar concentrations did not influence the activity of the horse serum which was used for the standard experiments reported in figure 1

The lymph flowed freely from the thoracic duct. A steady lymph flow from the cervical duct was insured by nodding movements, which were maintained manually at a rate of five to ten a minute. As soon as some lymph was collected in the tip of the cannula it was transferred by means of a pipette to a measuring cylinder Samples were used for the determination of their activity only if on simple observation they appeared to be free from blood Cervical lymph was usually perfectly clear and colorless, while thoracic lymph was often turbid and colored. The cholinesterase activity of blood serum, thoracic lymph, and cervical lymph was found not to change significantly during preliminary periods up to five hours of constant experimental conditions.

Blood samples were removed under liquid paraffin from a vein of a foreleg, stirred gently with a glass rod to remove the fibrin, and the serum obtained by centrifugation. Because we have found some evidence that the inhibitory effect of prostigmine (and physostigmine) diminishes when serum containing it is left for some time at room temperature, samples not analyzed at once should be kept below 10 C The experimental procedure is illustrated by table 2 which represents a typical experiment.

The results of ten experiments similar to that shown in table 2 are summarized in table 3 It is apparent that there is in lymph an enzyme which behaves like cholinesterase. Furthermore the activity of the esterase in lymph is less than in serum, but the former does not seem to bear any quantitative relation to the latter The enzyme activity of cervical lymph was equal to that of thoracic lymph in one out of seven experiments while in the other experiments it was distinctly less

It has been shown that cholinesterase is sensitive to certain inhibiting poisons especially to physostigmine and prostigmine (4 6 15) Therefore in order to characterize the enzyme further we have examined the influence of prostigmine on the activity of the esterase in thoracic and cervical lymph (see tables 2 and 3) Prostigmine methylsulfate was administered by stomach tube or by intravenous injection The doses of prostigmine used in

[3] Heparin used was obtained from the Connaught Laboratories of the University of Toronto.

these experiments (see table 3) did not cause either a marked or a consistent change in lymph flow Invariably prostigmine caused a decrease in enzyma-

TABLE 2

Dog no 9, 15 5 kgm, female Spontaneous respiration

July 10, 1940 5 00 p m 750 cc water by stomach tube
July 11, 1940 8 30 a m 500 cc water by stomach tube
9 00 a m 0 033 gram Nembutal per kgm i v
9 30 a m Operation started
9 34 a m 0 25 gram Nembutal i v
11 10 a m Collection of thoracic and cervical lymph started

TIME	SERUM	THORACIC LYMPH		CERVICAL LYMPH		REMARKS
		Sample volume	CO_2 mM/l	Sample volume	CO_2 mM/l	
	CO_2 mM/l.	cc.		cc.		
11 10		0		0		
11 29	6 2					
11 38		10 0	3 4			
11 46						Nembutal 0 1 gram
11 52				5	1 0	
11 56		10 0				
12 22		10 0				
12 40						Nembutal 0 1 gram
12 55	6 0					
12 58		10 0	3 0			
12 59				3 7	1 1	Prostigmine methylsulfate 0 5 mgm
1 47	2 7					
1 55						Nembutal 0 1 gram
2 07		20 0	1 3			
2 33	2 7					
2 43				5 0	0 3	
2 48		10 0	0 6			
3 38	3 6					
4 07		15 0	1 7			
4 28						Nembutal 0 1 gram
4 37				5 0	0 5	
4 55		10 0	2 2			
5 04	4 2					
5 08						Prostigmine methylsulfate 4 mgm
5 21		10 0				
5 28	0 7					
5 30		7 0	0 1	2 4	0 1	

tic activity, thus further indicating that the enzyme involved is cholinesterase In table 3 the titers of activity after prostigmine refer to the lowest value obtained, irrespective of time As can be seen from the experiment reported

in table 2, the effect of prostigmine was already maximal in Sample 4 of thoracic lymph and in Sample 2 of cervical lymph. Thereafter the enzyme activity in the serum and in the lymph increased very slowly, and in this experiment the activity had not yet returned to normal five hours after the injection of 0.5 milligrams of prostigmine. Whereas the average activity of serum cholinesterase after prostigmine fell in our experiments to 43 per cent of the original value the average activity of the cholinesterase in thoracic and cervical lymph fell to 33 and 26 per cent respectively.

In the experiments shown in table 3 the thoracic lymph flow, calculated from the total amount collected during the whole period of the experiment amounted to an average value of 24.2 cc per hour (range 12.7 to 49.2) and

TABLE 3

Dogs. Cholinesterase activity of serum, thoracic and cervical lymph before and after prostigmine

DOG			ACTIVITY SERUM	ACTIVITY THORACIC LYMPH	ACTIVITY CERVICAL LYMPH	PROSTIGMINE	AFTER PROSTIGMINE		
Number	Weight	Sex					Activity serum	Activity thoracic lymph	Activity cervical lymph
	kgm.		CO_2 mM/l.	CO_2 mM/l.	CO_2 mM/l.	mgm	CO_2 mM/l.	CO_2 mM/l.	CO_2 mM/l.
1	31.4	M	8.4	4.6					
2	13.6	M	5.9		1.6				
3	17.5	F	8.7	5.4		10*	5.8	4.6	
4	26.4	F	11.2	8.6	4.8	20*	4.6	4.3	2.1
5	14.8	F	5.7	4.4		30	0.9	0.5	
6	18.0	M	11.2		1.4	0.3	5.6		0.3
7	18.1	F	10.8	3.3	2.0	0.4	5.6	0.9	0.7
8	12.0	M	6.0	3.4	3.4	0.4	2.3	0.6	0.2
9	15.5	F	6.1	3.2	1.1	0.5	2.7	0.6	0.3
10	27.4	M	7.4	4.5	2.1	1.0	3.1	0.9	0.4
11	22.6	M	9.6	6.6	1.9	1.0	3.1	1.6	0.1
12	26.0	M	7.2	4.6	1.9	1.0	2.2	0.5	0.5

* Prostigmine administered orally

to an average value of 1.3 cc per hour (range 0.7 to 2.8) for every kilogram of body weight. It is obvious therefore that an appreciable amount of cholinesterase is continuously entering the blood stream with the lymph especially the thoracic lymph.

SUMMARY

1. A manometric method for the determination of the activity of cholinesterase in body fluids and tissue extracts is described. Of the species of animals examined, the activity of cholinesterase is highest in the serum of horse and man, lower in the dog, and lowest in the cat.

2. The cholinesterase activity of thoracic lymph and cervical lymph of

dogs, per cubic centimeter, is distinctly less than the cholinesterase activity of serum

3 Prostigmine methylsulfate given by mouth or by intravenous injection inhibits the activity of the cholinesterase in thoracic and cervical lymph

We are indebted to Dr Cecil K Drinker for demonstrating to us his method of collecting lymph

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THE RATE OF THERMAL DESTRUCTION OF WATER BALANCE PRINCIPLES IN PITUITARY (POSTERIOR LOBE) EXTRACT ASSAYED BY THE BURN AND BOYD-MACK METHODS

ELDON M. BOYD AND RODERICK W DINGWALL

From the Department of Pharmacology Queen's University Kingston, Canada

Received for publication January 6, 1941

Pituitary (posterior lobe) extract, the pharmacopoeial extract (B.P and U.S.P) of the posterior lobe of the hypophysis has a number of effects upon water metabolism which may be due to the same or to different substances present in the extract In mammals there are the Magnus and Sharpey Shaeffer diuretic action and the Van den Velden antidiuretic action and these reactions are well known in their many ramifications. In birds (2), reptiles (1) and amphibia (4) the extract may also stimulate either loss or retention of body water depending upon the circumstances of the experiment.

To determine if these various effects are due to one or to more than one substance in pituitary (posterior lobe) extract it is first necessary to have quantitative methods of assay For mammals Burn's (5) modification of Gibbs method has become standard, it measures the delay in diuresis produced by injecting the extract into rodents to which water has been administered In amphibia, a method has recently been proposed by Boyd and Mack (3) it measures the uptake of water by frogs immersed in water and injected with the extract, a reaction known as the Brunn reaction and which is due to an inhibition of excretion of water by the extract in frogs In the Burn assay the primary site of action of the extract is believed to be upon the kidneys, possibly upon the thin segment of the loop of Henle, in frogs, the primary site of action is believed to be extra renal, probably upon the muscles, subcutaneous and other tissues of the body Although these two reactions the Van den Velden and the Brunn reactions have some points in common, it is generally held that they are due to two different substances chiefly because pressor fractions of pituitary (posterior lobe) extract contain per milligram of original tissue more of the mammalian diuresis-inhibiting factor than oxytocic fractions while oxytocic fractions contain more than pressor fractions of the principle responsible for the Brunn reaction (11) In the investigation herein reported evidence was sought by another method to prove that these reactions were due to the same—or to different—substances.

The method employed has been recently used by Heller (7) to determine if

the pressor and antidiuretic principles are separate or identical entities Heller placed ampules of pituitary (posterior lobe) extract in boiling water for varying periods of time at various hydrogen ion concentrations, then measured the percentage loss of antidiuretic and pressor activity and found the antidiuretic factor more stable than the pressor factor Because of this, Heller concluded that the two principles were probably distinct and different substances

Using a procedure similar to that of Heller, we heated ampules of Pituitrin Surgical (Parke, Davis and Co) in a gently boiling water bath for periods varying from 30 minutes to two days We then assayed these various heated extracts in albino rats for diuresis-inhibiting factor by the method of Burn (5) with some of the modifications suggested by Gilman and Goodman (6)

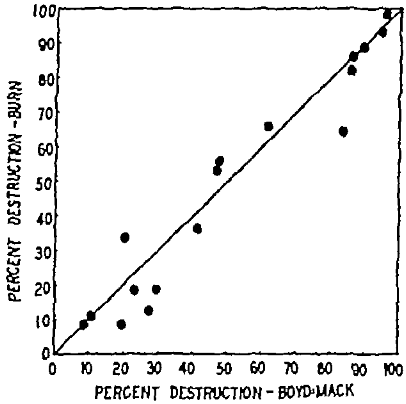

FIG 1 A COMPARISON OF THE PER CENT DESTRUCTION BY HEAT OF PRINCIPLES RESPONSIBLE FOR THE BURN AND BOYD-MACK ASSAYS OF WATER BALANCE ACTIVITY IN PITUITARY (POSTERIOR LOBE) EXTRACT

The extracts were also assayed by the method of Boyd and Mack (3) The amount of each of the principles responsible for these assays found in heated extracts was compared with the amount present before heating and the per cent destruction of active material calculated

The per cent destruction of activity indicated by the Burn assay was then compared with the per cent destruction of activity shown by the Boyd-Mack assay, the results have been plotted in figure 1 As might be expected, due to the error of 25 per cent or so in both methods of assay, there was considerable variation, especially in the extracts heated for short intervals so as to destroy from 10 to 30 per cent of activity The results agreed more closely when a large amount of activity had been destroyed, especially over 80 per cent When all of the data were plotted they fell reasonably well about a theoretical line, indicating that one principle was destroyed as rapidly as the

other, as may be seen in figure 1 The coefficient of correlation of per cent destruction by the two methods, calculated after the formula of Wyllie (12), was +1 0 with a probable error of 0 16 which indicates perfect correlation

From these results it is evident that the principle in pituitary (posterior lobe) extract responsible for the Burn assay is destroyed by heat at the same rate as the principle responsible for the Boyd Mack assay By comparing rates of thermal destruction it has not been possible to demonstrate that the principles responsible for these assays are separate entities In fact, the data may be offered as collateral though of course not conclusive evidence that a single substance effects both reactions

The chief evidence against the latter view is that the method of Burn reveals more activity in pressor than in oxytocic fractions of pituitary (posterior lobe) extract while the converse is true of the Boyd Mack method We have confirmed this anomalous situation in this laboratory by assays of pitressin and pitocin. It would not seem that extraneous matter in, or methods of separation of pitressin and pitocin are responsible for this difference since Oldham (9) obtained a more pronounced Brunn reaction in Stehle's postlobin-O than in postlobin V and Stehle's fractions (10) are made by a method quite distinct from that of Kamm et al (8) for pitressin and pitocin

A further fairly considerable body of evidence might be reviewed at this juncture for and against the argument that the principles responsible for the Burn and Boyd Mack assays are identical or distinct. The only conclusion which may be drawn at present, however is that while the available data do not prove the principles identical, neither do they prove them to be distinct. Evidence presented by the present report would tend to favour the former view

CONCLUSION

It was found that principles responsible for the Burn (5) and Boyd-Mack (3) assays of water balance activity in pituitary (posterior lobe) extract are destroyed by heat at the same rate

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THE BIOLOGICAL ESTIMATION OF ERGOMETRINE

EDWIN J DEBEER AND PAUL E TULLAR

From The Burroughs Wellcome & Co U S A Experimental Research Laboratories, Tuckahoe, N Y

Received for publication January 11, 1941

In studying various methods for the biological estimation of ergometrine, we had an opportunity of investigating several of the more promising but as yet undeveloped possibilities Among these was a suggestion by Rothlin (1) that the hyperthermia produced by the drug in rabbits be used as a method of its estimation The mydriatic effect also was studied since preliminary experiments suggested that this effect appeared to be proportional to the dose Either of these possibilities constitute a somewhat different angle of approach since previous biological assay studies appear to have been restricted largely to the action of this drug on the uterus, the intestine or the cock's comb A review of the literature gives the impression that no matter what the test object may be, the assay is still in the formative state For instance, Burn (2) in 1937 stated, "There is no biological method for estimating ergometrine available at the present time" Swanson, Hargreaves and Chen (3) recommended that the bioassay on the isolated rabbit's uterus be supplemented by colorimetric and polarimetric determinations and finally by assay on the postpartum human uterus The possibility of adding either the mydriatic or hyperthermal effects to the present biological assay armamentarium depends upon the extent to which various biological assay requirements are satisfied The usefulness of such an assay will depend upon other considerations, especially the correlation with clinical findings

METHODS

After some preliminary work, the following experiment was carried out in order to secure data from which possible dose-response curves might be obtained for statistical examination Graded doses of ergometrine acid maleate dissolved in physiological saline solution were injected in the marginal ear veins of 6 groups of albino rabbits, each group containing 4 animals Rectal temperatures were measured with a thermometer and the pupil diameters with a millimeter scale, immediately before the drug was injected and at frequent intervals thereafter Readings on both right and left pupils were taken and averaged The importance of the units of measurements may be emphasized here If, for instance, the temperature increases had been expressed in °F instead of °C, values for the slope, standard deviation and relative results would have been quite different

RESULTS

It was found that both the hyperthermal effect and the mydriatic effect are well suited for quantitative bioassay purposes since, as figure 1 shows, two well defined dose-response curves were obtained, relating log dose and the increase in rectal temperature in the one case and log dose and the increase in pupil diameter in the other The results of a statistical examination of

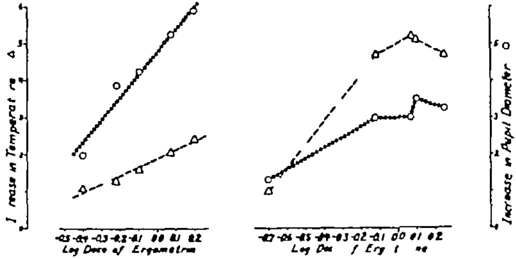

FIG 1 DOSE-RESPONSE CURVES FOR ERGOMETRINE AND ERGOTOXINE
The dotted lines represent the mydriatic effect and the dashed lines represent the hyperthermal effect. The positions of the ergometrine curves were calculated by the method of least squares

TABLE 1
Increases in rectal temperature and in pupil diameter following various doses of ergometrine acid maleate

DOSE	INCREASE IN PUPIL DIAMETER (mm.)				INCREASE IN RECTAL TEMPERATURE (°C.)			
mgm./kgm.								
0 4	3 0	1 0	1 5	2 5	1 1	0 8	1 2	1 2
0 6	3 0	2 5	5 0	5 0	1 5	1 1	1 0	1 5
0 8	3 5	4 5	5 0	5 0	1 5	1 9	1 6	1 3
1 2	5 0	5 0	5 5	5 5	2 2	2 1	2 3	1 6
1 6	6 0	4 0	6 5	5 0	3 3	2 2	2 6	1 1
1 6	7 0	6 5	6 5	5 0	2 4	2 7	2 6	2 5

the data of table 1, using standard statistical methods such as described by Snedecor (4) are given in table 2 In each case the strongest correlations were found when the maximum response observed was used instead of the response measured at some given time interval after injection such as one hour The high correlation coefficients and the correspondingly low values of P make it extremely improbable that the ergometrine curves of figure 1 are

the results of pure chance or accident Each curve can be considered a straight line since the deviations from linearity were not significant when the variations in animal response to a given dose are considered This was determined by calculating F, the ratio of the mean square of the deviations from linear regression to the mean square of the deviations within groups and finding that in each case the F value was less than the tabular value for the 5 per cent point This is true for the dosage range employed, i e, 0 4 to 1 6 mgm per kilogram Preliminary work indicated a tendency to form plateaus at higher doses As table 2 indicates the curves are sufficiently steep and the standard deviations are small enough so that even when a relatively small number of animals are used the limits of error are narrow enough to compare favorably with those of the more accurate biological

TABLE 2

Statistical analysis of the characteristic curve data

	MYDRIATIC EFFECT	HYPERTHERMAL EFFECT
Correlation coefficient	r = 0 847, P = <0 001	r = 0 803, P = <0 001
Deviation from linearity (F = 2 93 when n_1 = 4 and n_2 = 18)	F = 1 319	F = 0 203
Slope	b = 6 09	b = 2 36
Standard deviation of response	s = 0 886	s = 0 408
Limits of error as per cent of the mean potency	85–118%	82–122%

The low values of P indicate that the correlations are very strong Since the values of F are considerably less than the tabular figure of 2 93 for the 5 per cent point, the deviations from linearity are not significant

Limits of error are calculated on the basis of twice the standard error and on the assumption that a total of 16 rabbits are used for the assay, i e, the upper limit = 100 antilog $\frac{2s}{b}\frac{1}{\sqrt{16}}$ and the lower limit = 100 1/antilog $\frac{2s}{b}\frac{1}{\sqrt{16}}$

assays Of course, by using larger numbers of animals, the limits of error would be made still narrower

DISCUSSION

Specificity

Many biological assays such as those depending on LD 50 values, the contraction of smooth muscle, the increase in blood pressure, etc, are more quantitative than qualitative since more than one drug may produce essentially the same measured effect An assay based on either the hyperthermal or the mydriatic effect alone would fall into this class When, however, both responses are considered together an interesting problem in probability arises which serves to give the assay certain qualitative aspects In order for some other drug or mixture of drugs to give the same results as ergometrine, the

following specifications would have to be met (a) both mydriatic and hyperthermal effects must be produced (b) the types of characteristic curves must be the same (c) the ratio of the mydriatic response to the hyperthermal response at any given dose must be the same for either drug, (d) the potencies for each effect must the same While it is quite possible that one or two of these criteria might be fulfilled, the chances become quite small that all will be satisfied However, sufficient animals would have to be used so that the position of each curve is reasonably well fixed in order for specification (c) to be useful and the value of specification (d) becomes less as the difference between potencies becomes less.

As a practical example ergotoxine, a drug which might be expected to closely resemble ergometrine has been studied for the sake of comparison The results are shown in figure 1 It will be seen that specification (a) and probably (b) are met Quite different results are experienced when specification (c) is applied With ergometrine the mydriatic response (measured in mm.) is about 2.5 times greater than the hyperthermal response (measured in C) With ergotoxine conditions are reversed and using the steeper portion of the curves, the mydriatic effect is less than the hyperthermal, the ratio at log dose = -0.4 being about 0 7 Specification (d) is of less qualitative value since the relative potencies of the two drugs are not widely different. However it is evident that smaller doses of ergotoxine than ergometrine are required to establish the sub-plateau portions of the various log dose-response curves In this particular example aside from the qualitative features discussed above, ergotoxine was found to have a greater lethal action than ergometrine only those animals receiving the smallest dose surviving No animals were killed with the ergometrine. Also, other more subjective symptoms were different. When all of the above points were considered, the results with ergometrine could be distinguished easily from those of ergotoxine Mixtures of ergometrine with ergotoxine or with any other drug producing ergometrine-like effects would have to be separated before assaying

Sources of error

Aside from the inherent error due to animal variation, environmental influences may affect the absolute values observed. The room temperature may alter the hyperthermal response. Also it was evident that accommodation of the pupil to light was not abolished with smaller doses of ergometrine. Therefore although daylight was used in these studies some constant source of light would be more desirable

No differences attributable to sex were discerned

Design of the assay

Several types of assay design are possible, although in all cases a standard curve should be determined for each assay since according to the literature,

considerable differences in response to ergometrine are to be expected between species and strains Absolute values, therefore, would be difficult to reproduce Either the mydriatic or the hyperthermal curves may be used separately In this case the steeper of the two dose-response curves will give the more accurate results Efficient statistical handling of this type of assay is described by Bliss and Marks (5) If the results of both mydriatic and hyperthermal effects are to be combined, considerably more elaborate treatment would be necessary It was found that a certain degree of tolerance to ergometrine is produced by a single dose This fact might eliminate the possibility of using the "cross-over" technique

SUMMARY

It was found that both the mydriatic and the hyperthermal effects produced by ergometrine in the rabbit are quantitatively related to the dose and thus either may serve as a basis for the quantitative estimation of this drug

When both of these effects are considered simultaneously qualitative properties are acquired in addition It is then possible to distinguish between the results produced by ergometrine and those of ergotoxine

Effective doses of ergometrine were found to be less toxic than those of ergotoxine

Using 16 rabbits for an assay, limits of error of about ± 20 per cent may be expected.

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PHARMACOLOGIC RESPONSES OF DAPHNIA MAGNA

TORALD SOLLMANN AND WALTER WEBB

From the Department of Pharmacology of the School of Medicine of Western Reserve University Cleveland, O

Received for publication November 13 1940

Daphnia magna is a small transparent crustacean which can be easily reared in unlimited quantity It has the highly differentiated organ structure of crustaceans and its transparency permits direct visual observation of all of its internal as well as external movements under a low power microscope. This suggested the possibility of its use as a convenient object for the preliminary general survey and perhaps localization of pharmacological actions, if these are sufficiently similar to those of vertebrates Daphnia has been used by Viehoever (4 5) with apparent success for the bio-assay of a considerable number of drugs It therefore appeared interesting to make a study of the reactions of this animal to certain drugs that have characteristic actions. The summary of the results at the end of this paper indicates that the responses of this organisms differ rather widely from those of vertebrates. The usefulness of the animal for pharmacologic orientation in this direction therefore is limited The results are perhaps all the more interesting on their own account.

METHODS

The daphnias used in the following experiments were taken from a mass culture not graded as to size or age Daylight was used for observation. A single daphnia was placed on a hanging-drop slide and observed with the low power objective Drugs in solution were added an excess of fluid being avoided to facilitate keeping the animal in the field. The observations included the heart, respiratory appendages intestines, antennae, tail mandibles and eyes Gross movements were observed by placing daphnia in test tubes containing solutions of drugs representative of various pharmacologic groups.

RESULTS

The heart

The response of the daphnia heart to autonomic drugs indicates the presence of sympathomimetic action but fails to establish true vagomimetic or vagolytic action Epinephrine (1 10 000) in daphnia, as in vertebrates, causes a moderate increase in rate (20 to 40 beats per minute) Mecholyl (1 10 000) and pilocarpine (1 10 000) have no effect on heart rate but

physostigmine (1 10,000) causes a moderate slowing (20 to 40 beats) Atropine, used without other drugs, causes moderate slowing The antagonistic actions of the autonomic drugs were variable These responses are in agreement with Bonnet's (2) observation that the crustacean heart has sympathetic but no parasympathetic innervation Garrey (3) also noticed that the Limulus heart was not affected by parasympathetic stimulation Bain (1) records a marked acceleration of Cancer heart with epinephrine and a similar effect with pilocarpine, but no effect with atropine alone He also observed that atropine had no effect on the epinphrine response, but antagonized the pilocarpine effect

Those drugs which have their major action on cardiac muscle in vertebrates show a similar but not entirely characteristic response in daphnia Digitalis causes a slowing of the heart and arrythmias of dropped beats, from which the animal generally recovers if the digitalis solution is removed There is also a diastolic dilatation The slowing and arrhythmia correspond to the action of digitalis in vertebrates Barium (1 1000) in vertebrates causes a slowing similar to digitalis In daphnia this effect develops slowly and there is slight dilatation but no arrhythmia Quinidine (1 500) in daphnia causes a slow arrhythmia which is very irregular, with incomplete systole and diastolic dilatation Quinine (1 500) has a similar effect in slowing the heart and causing slight dilatation, probably by protoplasmic depression Magnesium (1 100), as a representative direct muscle and nerve depressant, causes slowing and arrhythmia with incomplete systole and diastolic dilatation

As a representative of the general protoplasmic depressants, ether (saturated aqueous solution) causes the heart to slow with slight dilatation and arrhythmia Alcohol (1 per cent) causes slowing and arrythmia These actions correspond fairly well to the depressant actions in vertebrates Pentobarbital (1 500) causes slowing, but no arrhythmia

Three types of asphyxiants were tried Carbon dioxide administered in saturated aqueous solution, sodium cyanide (1 10,000), and tannic acid (1 per cent) which, by precipitation of protein on the gills, prevents gaseous exchange In all cases the heart was slowed but its beats remained rhythmic Presumably the action is a direct cardiac muscle depression similar to that occurring in vertebrates

The remaining drugs tried give rather confused effects on the vertebrate heart, and are, therefore, not well suited for comparison They produce the following results in daphnia *Simple slowing* Cocaine (1 200), Metrazol (1 100), guanidine (1 500) develops slowly, strychnine (1 10,000), nicotine (1 1000) after primary increase, $BiMe_3$ (sat aq sol) after primary increase *No constant effect* Curare (sat aq sol), Veratrine (1 100) *Quickening* Nicotine, $BiMe_3$ Nicotine produces first a moderate increase in rate, then a progressive decrease Bismuch trimethyl causes immediate increase in rate which progressively slows

In general, daphnia magna does not appear to be a very useful reagent for predicting the action of drugs on the vertebrate heart. It agrees for sympathetic stimulation (epinephrine) and for muscular depression both direct (guanidine magnesium, ether barbiturate) and asphyxial (carbon dioxide cyanide, tannic acid) but it does not agree for parasympathetic stimulation (mecholyl) or for cardiac tonics (digitalis, barium)

Peristalsis

Daphnias generally show very little peristalsis but when it occurs there are deep contraction bands which move along the gut caudally causing expulsion of feces from the anal orifice. The dilated portion of the gut near the anus may contract without visible activity elsewhere In general the autonomic drug responses in daphnia are similar to but not identical with those seen in vertebrates

Epinephrine inhibits peristalsis when present. It also antagonizes the peristalsis started by mecholyl However epinephrine does not cause relaxation of the gut as it does in vertebrates Mecholyl pilocarpine and physostigmine produce marked increase in peristalsis and expulsion of intestinal contents Physostigmine tends to cause spasm. Atropine diminishes the spasm and inhibits the action of mecholyl and pilocarpine. These actions of parasympathetic stimulants on the gut are in striking contrast to their lack of action on the heart Nicotine first causes some increase in peristalsis which is followed by arrest

The muscular stimulants cause varying actions on the gut Barium causes slow peristalsis which is not violent and does not end in spasm. Digitalis causes no peristalsis. Cascara causes an increase in peristalsis in some and in others has no effect.

The muscular depressants with the exception of ether and alcohol have actions similar to those in vertebrates. Magnesium causes some slowing of peristalsis, ether increases peristalsis alcohol causes slight increase of peristalsis quinine and quinidine cause decrease in peristalsis.

Of the asphyxiants carbon dioxide causes no change in peristalsis cyanide causes cessation, and so does tannic acid

Certain drugs which have confused actions on vertebrate peristalsis cause the following effects on daphnia peristalsis *Increase* Strychnine (increases then decreases) Guanidine BiMe₃. *Variable* Pentobarbital Curare Veratrine. *Decrease* Metrazol Cocaine

It appears therefore that the intestinal responses to drugs with sympathetic and parasympathetic actions are similar in daphnia and in vertebrates so also is the response to cascara. With many other drugs however the effects do not correspond so that daphnia is not a reliable test object for peristaltic action in general

Striated muscle

Voluntary muscle reactions are complicated by direct and reflex control as well as peripheral actions In daphnia the muscles of respiration, antennae, and eye have a rhythmic type of movement, while the tail movements are essentially non-rhythmic The antennal movements are more jerky and are modified by the surroundings When free-swimming, the movements are quite continuous with a periodic rhythm (2 to 3 beats, a pause, then 3 to 4 beats, etc), but at varying rates and magnitudes Perhaps 80 to 120 beats per minute are normal rates When the animal is confined to a hanging drop slide the same type of rhythm and movement is maintained, but the rate is slower (20 to 40 per minute) The eye normally has a rapid vibratory movement and rotates quickly through a small angle This rhythm is constant and seldom broken When the eye is disturbed it generally loses the rapid vibratory movement and assumes slower rotation through a larger angle The tail movements are periodic rather than rhythmic At times the tail is used to assist in propulsion Frequently it moves to clean the gills with the hook on its end After feces have been expelled the tail moves the material away so that this is not carried up into the gills The movements are normally quite vigorous and at a rate of about 10–20 per minute The mandible moves quite rhythmically, 80–120 times per minute, in a grinding fashion The activity is increased when food passes into the gullet These structures, representing various types of rhythmic and nonrhythmic muscular movements were observed individually, but as their responses are generally similar they will be discussed together, except where significant differences occur The respiration will also receive separate consideration

Curare produces first increase then decrease and finally arrest of all movements After all movements had ceased, electrodes were applied directly to the shell of the animal and a single break shock given The result was a single contraction of the tail, a few beats of the respiratory appendages, and a single beat of the antennae No movements of the eye or mandibles were noticed The same experiment was tried following magnesium, ether, and nicotine, but no movements occurred with stimulation The fact that the animal responded to artificial stimulation after all normal voluntary activity had ceased suggests that the muscle is still irritable and that the action may be on the neuro-receptive mechanism as in vertebrates

Agents which produce muscular twitchings in vertebrates by peripheral action have equivoval actions in daphnia Barium with free swimming daphnia, causes first increased activity with normal periodic movements of the antennae This is followed by decreased activity On the hanging drop slide there are occasional vigorous movements of the tail, the mandibular movements become slower and irregular, the antennae showed fine twitchings, the eye stalk movements change from continuous rapid vibrations to slower, irregular, larger angle rotation and trembling All the above reactions correspond closely to the fibrillary twitchings seen in vertebrates Guanidine showed no movements of the tail and arrested the movements of the antennae, mandible, and eye stalk, all of which seems opposite to the action in vertebrates Mecholyl, pilocarpine and physostigmine apparently produced little or no well defined effects on the muscular movements of

daphnia, in contrast to the twitching and contracture of vertebrate skeletal muscle and the observation of Bonnet (2) that mecholyl prevents the paralyzing effect of strychnine on crayfish Nicotine produces marked increase of muscular activity in free-swimming daphnias so that they circle and loop rapidly On the hanging-drop slide the antennal movements show some increase in rate and tendency toward continuous rhythm. Later the amplitude is decreased and the tail assumes a characteristic position There is no indication of twitching or tonus Epinephrine which probably has no effect on the muscular movements of vertebrates seems to cause stimulation in daphnia, manifested by increased rate of antennal movements and more continuous rhythm but decreased amplitude The tail also shows increased rate but decreased amplitude and changes to continuous type of movements. Eye stalk movements become large angle, rotatory movements. Here again free swimming daphnia show a tendency to more rapid and continuous antennal movements with looping and circling Quinidine causes slow vigorous movements of the tail, slow, irregular movements of the mandible and continuous jerking of the antennae while the eye movements are not changed. Quinine causes no change in tail movements decrease in amplitude and twitching antennal movements, slow movements of the mandible and no change in eye movements. Veratrine causes slow movements of the tail, but there is no noticeable lengthening of the relaxation as in vertebrates. There are variable but generally slow movements of the mandible slow feeble movements of the antennae, and a variable action on the eye stalk.

The agents whose action in vertebrates is chiefly depressant, either central or indirect, elicit similar results in daphnia Ether in free-swimming daphnia, causes a very brief increase in activity followed by a marked decrease On the hanging drop slide the tail shows weaker rapid trembling movements the mandibular movements cease the antennal movements increase in rate followed by slowing their activity becomes tremulous and finally stops entirely The effects correspond chiefly to narcosis.

Alcohol appears to cause some excitation before depression. On the hanging drop slide, tail movements are first increased in rate and amplitude but later decreased Eye movements are generally slow rotation through a large angle Antennal movements are first increased, then become gradually slow, small in amplitude and tremulous On free-swimming daphnia, alcohol causes some increase in activity but no tendency to circle or rotate This is followed by a progressive decrease and cessation of movements Pentobarbital appears to act chiefly as a depressant. Magnesium has rather slight effect.

The asphyxiants in daphnia, as in vertebrates, cause convulsive movements followed by depression Cyanide in free swimming daphnia causes an immediate increase in activity The antennal movements are of normal rhythm and there is no tendency to loop or rotate. Later there is a progressive de-

crease in activity With the hanging drop slide the tail movements are increased in rate and amplitude The antennae show rapid movements of a normal type and the eye stalk movements are slow, small-angle rotations Tannic acid causes both stimulation and depression, but the stimulation might well be sensory Carbon dioxide produces chiefly depression, the movements are quickly decreased and the animal sinks to the bottom of the tube In the hanging-drop slide, tail movements are slow and feeble, and eye movements cease

As the action of *convulsants* in vertebrates is associated with definite structures in their central nervous system which are quite different from those of the invertebrate ganglionic centers, the effects were watched with special interest Strychnine in free-swimming daphnia causes rapid, continuous beating of the antennae which results in rapid circling and looping The movements gradually become slower but remain of the continuous type, the amplitude being decreased The tail movements are decreased in amplitude and rate and the tail death assumes a characteristic position extending in the axis of the body Bonnet (2) noted that strychnine causes paralysis in crayfish and that this action is central Viehoever (4, 5) described effects in free swimming daphnia which he classes as convulsive Metrazol produces gradual depression of free-swimming daphnia, apparently without preceding stimulation On the hanging-drop slide, the antennal, mandibular and caudal movements are decreased in amplitude and increased in rate Cocaine causes complex motor effects The tail movements are not affected, but the mandibular movements become slow, regular, and feeble The antennal movements are continuous and jerky The eye movements stop immediately We may conclude that the drugs which are convulsant for vertebrates do not produce analogous effects in daphnia, though strychnine does show some phenomena of stimulation

Some miscellaneous drugs give the following results Digitalis is depressant It causes feeble movements of the tail, active mandibular movements, but cessation of antennal and eye movements Bismuth trimethyl results in rather marked stimulation followed by depression The free-swimming daphnia show increased movements, and the antannae beat with normal type rhythm There is no tendency to loop or rotate On hanging drop slides, tail movements are increased in rate and amplitude immediately, but quickly show decrease in amplitude, becoming weak and slow

Respiration

The respiratory movements consist of synchronous rhythmic sweeping movements of the ventral appendages, which are modified gill-like structures Occasionally the movements cease momentarily, then resume the rhythm of approximately 140 to 200 beats per minute This rhythm must depend upon the state of both the nervous centers and the peripheral muscular mechanism

Its response to drugs is very similar to that of the other striated muscle except that the stimulant phase of nicotine, epinephrine, cyanide and strychnine is absent, these depress respiration directly Drugs which depress vertebrate respiration also depress the respiration of daphnia, but the drugs which stimulate vertebrate respiration viz nicotine carbon dioxide cyanide, metrazol and strychnine, are purely depressant to the respiration of daphnia.

SUMMARY AND CONCLUSIONS

Attempts were made to determine the usefulness of daphnia magna as a suitable animal for preliminary pharmacological orientation The responses were as follows, as compared with vertebrates

Sympatheticomimetic drugs cause acceleration of the heart but only part of the other reactions typical for vertebrates

Parasympatheticommimetic drugs stimulate the intestines but do not slow the heart. Their intestinal action is prevented by atropine, as in vertebrates

Cardiac tonic drugs cause slowing of the heart, but dilatation instead of tonus.

Cardiac depressants agree in general with the action in vertebrates Skeletal muscle curare action probably agrees with curare action in vertebrates

Muscular twitching by peripheral action corresponds in general with that action in vertebrates but is somewhat equivocal

Chemical rigor was not observed

Respiratory depressants correspond in general to the vertebrate action.

Respiratory stimulants do not correspond to the vertebrate actions

Central convulsant action does not correspond to the vertebrate action except for some similarity with strychnine

Central depressants correspond in general to the vertebrate action.

It is seen that the pharmacologic reactions of daphnia differ qualitatively from those of vertebrates in many particulars, which limit the usefulness of this animal as a pharmacologic reagent.

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THE EFFECTS OF SODIUM DIPHENYL HYDANTOINATE (DILANTIN) ON VITAMIN C LEVEL IN TISSUES AND VITAMIN C EXCRETION IN RATS[1]

MILES E DRAKE, CHARLES M GRUBER AND VICTOR G HAURY

From the Department of Pharmacology, Jefferson Medical College, Philadelphia, Pennsylvania

Received for publication November 16, 1940

A number of investigators have shown that certain drugs influence the excretion of ascorbic acid from the body (1) Gruhzit (2) studied the effects of sodium diphenyl hydantoinate on the blood ascorbic acid level in rats and reported negative results Kimball (3) and Kimball and Horan (4) write that 51 per cent of their patients receiving dilantin in the treatment of epilepsy showed a varying degree of hyperplasia of the gums Their treated patients with normal gums showed no vitamin C deficiency, whereas every patient with marked hyperplasia of the gums presented a definite reduction in blood ascorbic acid Recently Merritt and Foster (5) published a paper in which they express the belief that dilantin has no effect on the plasma ascorbic acid level in man Frankel (6) reported that the deficiency in ascorbic acid content of the blood parallels to a certain degree the amount of hyperplasia of the gums in dilantin treated patients

Our interest in this problem was aroused by the above conflicting reports

METHODS

Adult albino rats, kept on a vitamin C free diet (7), were used in this work One group of animals, which was used for the study of urinary excretion of vitamin C, was kept in individual metabolism cages These cages were placed over large glass funnels with a trap for collecting the feces Below the large funnel was placed a 500 cc Erlenmeyer flask which contained in some instances 10 cc of a 10 per cent metaphosphoric acid solution and in other instances crystalline metaphosphoric acid and mineral oil The urine was collected over a 24 hour period It was made up to 100 cc and the total amount of vitamin C excreted was determined by titrating the urine into a known amount of standardized *2-6* dichlorobenzenoneindophenol

The sodium diphenyl hydantoinate was dissolved in distilled water to which was added 2 or 3 drops of 4 *N* sodium hydroxide solution The drug was administered by stomach tube as a single dose of 500 mgm per kilogram Fourteen animals were used in this particular series and 28 experiments were performed

Forty-three rats were used for the study of vitamin C in which the tissues and urine

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[1] This investigation was made possible by a grant from Parke, Davis and Company for research in science

were pooled. These were placed on a diet free of vitamin C (7) and kept in groups of 5 to 7 animals in each cage. After the animals were on this diet for a week a single dose of 500 mgm. of dilantin per kilogram was given by stomach tube to 29 of the 43 animals the remaining 14 served as controls. Four days after the administration of the drug

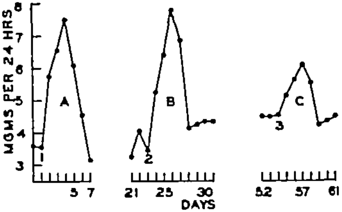

FIG. 1 shows the average daily urinary excretion of ascorbic acid in 14 rats. In curve *A* at 1 500 mgm. dilantin per kilogram were given by stomach tube. Twenty-three days later at 2 in curve *B* a second dose of 500 mgm. of dilantin per kilogram was given orally. Fifty-four days later at 3 in curve *C* a third dose of 500 mgm. of dilantin per kilogram was given the same animals.

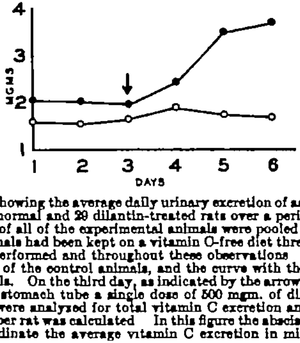

FIG. 2. A graph showing the average daily urinary excretion of ascorbic acid, in mgm. per 24 hours by 14 normal and 29 dilantin-treated rats over a period of six days. For analysis the urines of all of the experimental animals were pooled as were those of the controls. The animals had been kept on a vitamin C-free diet three weeks before these experiments were performed and throughout these observations. The curve with the open circles is that of the control animals, and the curve with the dots is that of the experimental animals. On the third day, as indicated by the arrow ↓ all of the treated rats were given by stomach tube a single dose of 500 mgm. of dilantin per kilogram. The pooled urines were analyzed for total vitamin C excretion and from these figures the daily excretion per rat was calculated. In this figure the abscissa indicates the time in days and the ordinate the average vitamin C excretion in milligrams per rat per 24 hours.

12 of the 29 experimental and 4 control animals were killed by cerebral concussion. One cc. of blood from each animal in the experimental group as it was killed was added to 20 cc. of a mixture containing 4 per cent metaphosphoric acid and 8 per cent trichlor acetic acid in water (8). Approximately 1 gram of liver was removed from each animal immediately after it was killed and this was frozen in a mixture of CO_2 ice and ether. Sections of muscle, brain and both adrenal glands were similarly treated. All sections

TABLE 1

The effect of dilantin given orally on the ascorbic acid content of certain tissues

The tissues and 1 cc of blood from each animal were analyzed separately and the results of all of the animals in each group were then averaged for convenience of publication One week was allowed between each administration of dilantin Seven of the animals designated † received 1 dose of dilantin before they were sacrificed, 6 marked ‡ received 2 doses and 5 indicated by * were given 3 doses

	NUMBER OF ANIMALS	TISSUES: Liver		Brain		Muscle		Adrenal gland		Blood	
		Ascorbic acid per gram	Per cent decrease	Ascorbic acid per gram	Per cent decrease	Ascorbic acid per gram	Per cent decrease	Ascorbic acid per gram	Per cent decrease	Ascorbic acid per 100 cc.	Per cent decrease
		mgm		*mgm*		*mgm*		*mgm*		*mgm*	
Controls	8	0 37		0 30		0 35		1 93		0 61	
Dilantin treated animals	7†	0 26	30	0 21	30	0 19	46	1 05	45	0 38	42
	6‡	0 25	32	0 19	37	0 15	56	0 98	49	0 22	60
	5*	0 25	32	0 10	65	0 09	76	0 72	64	0 22	60

TABLE 2

The effect of dilantin given orally (stomach tube) on the ascorbic acid content of certain tissues

The determinations were made on pooled organs

	NUMBER OF ANIMALS	TISSUES: Liver		Brain		Muscle		Adrenal gland		Blood	
		Ascorbic acid per gram	Per cent decrease	Ascorbic acid per gram	Per cent decrease	Ascorbic acid per gram	Per cent decrease	Ascorbic acid per gram	Per cent decrease	Ascorbic acid per 100 cc.	Per cent decrease
		mgm		*mgm*		*mgm*		*mgm.*		*mgm*	
Controls	14	0 38		0 30		0 37		1 93		0 73	
Dilantin 500 mgm /kgm body weight Animals killed 4 days later	12	0 28	26	0 19	35	0 20	46	1 10	43	0 38	48 5
Dilantin 500 mgm /kgm. and repeated 1 week later Animals were killed 4 days after 2nd dose	10	0 25	33	0 21	28 5	0 20	44 5	1 09	43	0 34	53
Dilantin 500 mgm /kgm followed 1 and 2 weeks later by 2nd and 3rd doses Animals killed 4 days after 3rd dose	7	0 26	31	0 19	37	0 24	35	1 12	42	0 36	51

of the same type of organ were weighed accurately while frozen and put into 20 cc. of the mixture of metaphosphoric and trichloracetic acids and ground with sand These pooled ground organs were then transferred to 50 cc tubes and centrifuged. The supernatant liquid was poured off made up to 50 cc and titrated with sodium 2-6 dichlorobenzenoneindophenol The tissues of the 4 control animals were treated in a like manner One week after the first administration of dilantin the 17 remaining experimental animals were given a second single dose of 500 mgm. of dilantin per kilogram and after a lapse of 4 days 10 experimental and 5 control animals were again sacrificed and the tissues treated as above One week after the second administration of dilantin the remaining 7 experimental animals were given a third single dose of 500 mgm of dilantin per kilogram. Four days later all of these as well as the 5 control animals were killed and the tissues analyzed

Twenty-six animals were used in a third series of experiments in which the vitamin C content of the tissues as well as that of the blood of each animal was determined separately Seven days after the animals were placed on the diet free from vitamin C 18 of them were given orally one dose of 500 mgm of dilantin per kilogram and four days later 2 of the animals which did not receive the drug and 7 which did were killed. Specimens of liver brain, skeletal muscle blood, and the adrenal glands of each animal were treated as above and analyzed for vitamin C content. The remaining experimental animals were given a second dose of dilantin one week after the first and four days later 6 of these and 3 controls were killed and the tissues analyzed The remaining 5 experimental animals were given a third dose of dilantin one week after the second and four days later all the remaining animals were killed and the tissues studied as above

RESULTS

Figure 1 shows the effects of single doses of dilantin on the average daily urinary excretion of vitamin C in 14 animals The drug was administered to the same animals on three different occasions which are designated 1 2 and 3 In this series of experiments the vitamin C excretion was determined daily for each animal and then averaged

It will be noted that the ascorbic acid excretion in the urine reached its peak within the first four days after the administration of dilantin. The first and second doses of dilantin (curves A and B) caused a greater excretion of vitamin C than did the third dose (curve C) Figure 2 shows a similar effect of dilantin on the daily excretion of vitamin C for six days in pooled urine from 29 animals.

It is seen from table 1 that all of the tissues studied show some decrease in ascorbic acid content following the administration of dilantin. In this series of experiments the adrenal glands, the brain and the skeletal muscle show the greatest reduction in vitamin C Table 2 shows the same general changes with pooled tissues.

SUMMARY

1. Sodium diphenyl hydantoinate (Dilantin) given orally to albino rats causes an increase in the excretion of ascorbic acid in the urine as determined by the sodium 2-6 dichlorobenzenoneindophenol method.

2 The maximum excretion of vitamin C in the urine following the oral administration of dilantin is reached on the fourth day, returning to its control level on the fifth to seventh day

3 The body supply of vitamin C appears to be gradually decreased by the administration of dilantin as indicated by our tissue studies

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THE PHARMACOLOGY OF SOME DERIVATIVES OF MONO ALPHA SECONDARY FURFURYLAMINE AND OF DI ALPHA FURFURYL TERTIARY AMINE

MICHAEL G MULINOS AND S. DISICK

From the Department of Pharmacology College of Physicians and Surgeons Columbia University New York

Received for publication January 13, 1941

The present report is concerned with the pharmacological properties of a series of furfuryl-amine derivatives synthesized and described by Zanetti and Bashour (1939 1940) No previous pharmacological investigation of these products could be found in the literature

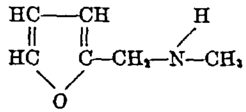
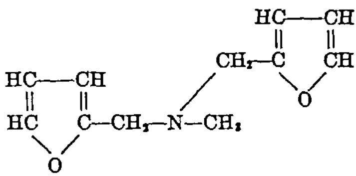
The parent substances for this series of furfuryl amines are *a.*—Furfurane *b*—Furfural *c.*—Methyl-furfurane and *d.*—Furfuryl-amine. McGuigan (3) found that furfural was slightly antiseptic that it was highly toxic to frogs resulting in convulsions, and that locally it was anesthetic In mammals the administration of furfural resulted in depression followed by paralysis and death He reviews the pertinent literature (see also 1)

Koch and Cohan (2) found that furfurane administered to rats and rabbits by inhalation resulted in convulsions of medullary origin followed by respiratory paralysis and death. Injection of 1 5 cc of furfurane into a dog weighing 10 kilograms caused immediate death. When the drug was given orally to rabbits death followed immediately in one case and in 24 hours in another Post-mortem examination revealed hyperemia of the lungs generalized venous dilatation and a cherry red color of the blood resembling that seen in cyanide poisoning

Fujii (1 b and c) reported upon the pharmacological activity of furfur-ethyl amine and of furfur methyl amine (furfuryl-amine) The precursor of the series under investigation is furfuryl-amine in which either one or both available hydrogens on the nitrogen are substituted. Fujii reported that furfur-ethyl-amine caused constriction of blood vessels by a direct action upon the smooth muscle. In the rabbit intravenous injections caused an immediate but transient fall in blood pressure followed by a rise which eventually ended in a permanent depression of the blood pressure. He reported depression of the isolated frog heart, while on the isolated rabbit heart there resulted an increase in the rate and strength of the beats. Isolated strips of intestine and of the uteri of rabbit guinea-pig and cat were stimulated by furfur-ethyl-amine.

The pharmacological action of furfuryl-amine was reported to be similar to that of the furfur-ethyl-amine Intravenous injection resulted in a fall of the blood pressure of the cat, while that of the rabbit was elevated There was little activity on the isolated frog heart and no depression of the central nervous system Isolated intestinal and uterine strips were contracted The M L D for mice was reported as 0 2 mgm per gram of animal

TABLE 1

DERIVATIVE OF MONO-ALPHA FURFURYL-AMINE	FORMULA	pH OF A 1 PER CENT SOLUTION IN 0 9 PER CENT NaCl SOLUTION	TEXT REFERENCE SYMBOL
-methyl		4 20	Z-1
-ethyl	$—C_2H_5$	4 15	Z-2
-n-propyl	$—C_3H_7$	3 95	Z-3
-n-butyl	$—C_4H_9$	4 10	Z-4
-n-amyl	$—C_5H_{11}$	3 82	Z-5
-phenyl	$—C_6H_5$	4 05	Z-6
DERIVATIVE OF DI ALPHA FURFURYL-AMINE			
-methyl		4 00	Z-7
-ethyl	$—C_2H_5$	4 00	Z-8
-n-propyl	$—C_3H_7$	4 10	Z-9
-n-butyl	$—C_4H_9$	4 12	Z-10
-n-amyl	$—C_5H_{11}$	4 18	Z-11

EXPERIMENTAL

Table 1 contains the formulae of the substances which we investigated

The hydrochlorides of all of these substances were soluble in 0 9 per cent NaCl except Z-6, which was dissolved first by adding several drops of concentrated hydrochloric (12N) acid, then neutralizing to pH 4 05 by means of sodium bicarbonate

When the fore-mentioned drugs were added to Ringer (pH 7 4) solution in the maximum concentration of 1 2,500 used, the reaction of the resultant solution lay between pH 6 7 and 7 1 The effects reported below were therefore not accounted for by changes in the reaction of the bath fluid

RESULTS

Isolated intestinal strips

Cat and rabbit intestine were suspended in Ringer's solution at 38 C in the usual manner Each of the preparations was instilled into the bath fluid at least once before any other medication. Where the drug had a definite pharmacological effect it was tried again in the presence of itself or in the presence of each of the others in order to detect any change in reaction due to such change in the environment.

Z-1, Z-2, Z-3, and Z-4 had no demonstrable effect upon the strips in concentrations from 1 5 000 to 1 ,50,000 while the reactivity of the strips to epinephrine, acetyl-choline and barium chloride was not in any way modified by the presence of these drugs. In like concentration the other members of this series from Z-5 to Z-11 inclusive depressed the rabbit intestinal strips Z-6 was the most active member of the group, as might be expected because it is a phenyl-amine derivative If the depression were not too great, acetyl choline and barium chloride were capable of stimulating the strip and epinephrine could depress it still further In no cases was the depression permanent, for the strip would recover satisfactorily after one or more washings with Ringer's solution.

Isolated uterine strips

Non-pregnant strips of the uterus of the cat, the rabbit and the guinea-pig and the strips of the pregnant cat and rabbit uterus were used. Z-1 to Z-4 inclusive had no effect on such strips The remaining drugs were divisible into 2 types, (*1*) Z-6 Z-9 Z-10 and Z-11 depressed the uterine strips to which they were added in concentrations of 1 100 000 or greater, Z-6 being the most powerful. The depression caused by these drugs was not permanent nor did their presence in the larger dilutions abolish the effectiveness of epinephrine, acetyl-choline, barium chloride or histamine phosphate Neither atropine nor ergotamine modified the reactivity of the uterus to them (*2*) Z-5 Z-7 and Z-8 had a stimulatory effect upon the non-pregnant rabbit uterus and no effect whatever upon the non-pregnant guinea pig uterus. Z-7 had no effect upon either the virgin or pregnant cat uterus Ergotamine tartrate had no effect on the action of Z-7 and abolished but did not reverse the action of Z-8 on the rabbit uterus.

Z-5 did not alter the activity of the non pregnant uterus of the cat but stimulated the pregnant organ. Ergotamine tartrate reversed the stimulatory action of Z-5 on the rabbit uterus once in 2 trials while on the pregnant cat uterus (which was consistently stimulated by Z-5) ergotamine invariably abolished although it never reversed the action This finding is consistent with the ineffectiveness of Z-5 upon the non-pregnant cat uterus which is normally depressed by epinephrine. These drugs did not abolish the re-

activity of the uteri to acetyl-choline or to epinephrine, nor did atropine modify the reactivity of the uteri to these drugs

The pupil of the rabbit was used in connection with Z-5 and Z-7 to test further the suggestion, implied by the foregoing facts, that these two drugs may possess "sympathomimetic" powers The drugs were instilled into the conjunctival sacs of normal rabbits and of rabbits from which the left superior cervical ganglion had been removed at least 6 months before No dilatation followed When injected intravenously in doses of 10 mgm per kilogram both compounds failed to dilate either the normal or the denervated pupil

Z-5 and Z-7, given to rabbits subcutaneously in doses of 10 and 20 mgm per kilo, failed to raise the blood sugar above that of the saline injected controls These same rabbits were found to be quite sensitive to the hyperglycemic effects of epinephrine in doses of 0 01 mgm per kilo

The isolated frog heart

The frog heart was perfused *a*—by way of the vena cava by a modified Loewi technic (4), or *b*—by way of the aorta according to the method of Straub (1910) The experiments were run at room temperature (22 to 23°C) using a two-thirds dilution of mammalian Ringer solution as the perfusate, which was aerated constantly during the experiment The hearts were removed from frogs which weighed between 45 and 60 grams after pithing and exsanguinating the animal The contractions of the perfused heart were recorded upon a kymograph by attaching either the ventricular apex alone, or both it and the auricle, to recording levers In this way we could determine the effect of the drugs upon the rate and strength of the ventricles and auricles and the effect upon A-V conduction

a—The Loewi preparation is more apt to respond to neuromuscularly acting drugs such as epinephrine and acetyl-choline because the perfusion fluid bathes the sinus venosus When an injection of one of the drugs was to be made the flow of fluid from the reservoir to the perfusion cannula was interrupted and the latter allowed to empty down to its narrow neck by the contractions of the heart At this time the cannula was filled with the solution to be tested In this way only a small sample of the proper concentration of drug was necessary—a procedure necessitated by the smallness of the samples available In this series Z-4, Z-7 and Z-8 were not tried due to lack of material

Z-1, Z-2, and Z-3 in concentrations of from 1 5,000 to 1 100,000 had no effect Z-5, Z-6, Z-9, Z-10 and Z-11 in similar dosage brought about slowing of the ventricular rate, weakening or stoppage Doses of atropine which abolished the bradycardia from acetyl-choline had no effect upon the depression from these drugs Only Z-9, in concentrations of 1 10,000 or larger, resulted in permanent cardiac arrest In these hearts epinephrine in concentrations greater than 1 10,000 always depressed the heart, but more dilute solutions were stimulant

The Z series of drugs had no obvious blocking action, the A V rhythm persisting unto complete stoppage of the heart.

b—The Straub method of perfusing the ventricle has the advantage that neural effects are minimal and in that very small amounts of drugs are necessary The column of fluid was fixed at 4 cm. above the aortic opening. Z-4 was not tried.

Z-1, Z-2 and Z-3 in concentrations as high as 1 1,000 were ineffective. Z-5 to Z-11 inclusive resulted in slowing, weakening or in stoppage of the heart depending upon the concentration. Usually upon washing with frog Ringer's solution, recovery was prompt and complete Z-9 Z-10 and Z-11 were the most toxic leading to persistent depression in concentrations of 1 5 000 Z-7 and Z-8 were the least toxic, being ineffective in concentrations less than 1 5 000 Epinephrine also depressed the heart in concentrations above 1 10 000 but it gave stimulation when more dilute. As before, atropine did not abolish the depression from these preparations.

The action on the blood pressure of the anesthetized cat

Adult cats anesthetized with sodium pento barbital 40 mgm. per kilogram intraperitoneally, were prepared for blood pressure recording by cannulization of the carotid artery, injections were made through a cannula into a saphenous vein The drugs were injected in doses of from 5 to 20 mgm. per kilogram usually as a 1 per cent solution and were washed in with 2 cc. of saline or Ringer's solution The animals were kept warm by means of an electric pad. Preliminary test injections of saline and of epinephrine usually 0 1 cc. of 1 10 000 per kilogram, and of small doses of acetyl-choline before and after atropine were made routinely Each animal was tested also with epinephrine at the end of the experiment

All of the drugs of this series that were tried caused a slight to a moderate depression of blood pressure, not abolished by atropine. From 10 mgm per kilogram or less the fall in pressure was slight and transitory though in one animal this dose of Z-10 caused the blood pressure to fall to zero this animal was revived by means of manual artificial ventilation but the blood pressure remained below the original level for the remainder of the experiment In doses of 20 mgm per kilogram Z-1 Z-2, Z-3 Z-5 Z-6 and Z-7 all caused a marked but transitory fall in pressure Z-5 being the most active. The effectiveness of epinephrine was not in any way modified by the injection of these drugs

Toxicity in frogs and mice

Frogs weighing 25 to 30 grams received these drugs into the anterior lymph sac in the usual manner Surviving animals were observed for 48 hours after the injection The frogs were kept in water at room temperature. The doses are reported in mgm per 10 grams of frog weight. Z-9 was not tried

In doses of 2 mgm. Z-5 caused definite depression lasting little more than 30 minutes, but Z-6, Z-10 and Z-11 caused excitement and hyper-reflexia up to 2 hours after the injection, Z-3 and Z-7 were ineffective Three to 4 mg of Z-1, Z-2, Z-3, Z-4 and Z-8 proved inert On the other hand, similar doses of Z-5, Z-6, Z-7 and Z-10 resulted in immediate generalized depression ending in death in from 10 minutes for Z-10 to 17 minutes for Z-7 Post-mortem examination revealed the heart stopped but irritable to electrical and mechanical stimuli, as were also the muscles and the sciatic nerves Z-11 resulted in depression followed by excitement, hyper-reflexia and tetanic convulsions These animals did not die A dose of 10 mgm of Z-3 caused depression within 15 minuted which deepened until death 2 hours later Autopsy revealed the heart still beating

Into albino mice weighing 30 to 35 grams these drugs were injected intraperitoneally as a 1 per cent solution and the doses are reported in mgm. per 10 grams of mouse The surviving animals were observed for at least 48 hours after an injection. Z-9 was not tried

In doses of less than 1 mgm Z-4 was ineffective, Z-5 resulted in convulsions in 2 minutes, followed by depression for an hour, with recovery Doses between 1 and 2 mgm of Z-1 and Z-2 were ineffective, similar doses of Z-3, Z-4 and Z-5 resulted in respiratory distress, ataxia, clonic convulsions and death in from 2 to 15 minutes Autopsy revealed the heart still beating feebly Corresponding doses of Z-6 gave rise to transient excitement whereas Z-7 and Z-8 caused immediate ataxia followed by generalized depression with death in 23 hours Post-mortem examination was negative Doses of from 3 to 4 mgm of Z-1 proved ineffective, 3–4 mgm of Z-2, Z-3, Z-4, Z-5, Z-7 and Z-8 led to ataxia, convulsions and death in from 2 to 10 minutes, and autopsy revealed the hearts still beating, 3–4 mgm of Z-6 resulted in immediate collapse with death in 22 hours Such doses of Z-10 and Z-11 elicited immediate convulsions lasting from 24 to 96 minutes The mice were exhausted but alive At this point, they were given another similar dose in order to test for the development of "tachyphylaxis" similar to that seen with nicotine Convulsions followed the second dose, the mice succumbing within 20 minutes Post-mortem examination was negative

SUMMARY

The foregoing data indicate that the series of furfuryl-amine derivatives listed has little pharmacological activity and that the order of toxicity is low The toxicity increases with the length of the substituents on the nitrogen molecule Four of the six mono-furfuryl-amines were less toxic than the di-furfuryl-amine derivatives

Amyl-furfuryl-amine, and methyl- and ethyl-di-furfuryl-amines stimulated the rabbit uterus and depressed the rabbit intestine, suggesting that they may possess some degree of "sympathomimicity" If so, the action is pre-

dominantly upon the motor mechanism because the effect upon the uterus is abolished but not reversed by ergotoxine and the non pregnant cat uterus is not inhibited. In this respect these substances superficially resemble ephedrine. On the other hand these drugs do not stimulate the frog heart nor do they raise but in large doses depress the blood pressure of atropinized cats. The other members of the mono-furfuryl-amines are inert while the others of the di furfuryl-amines depress smooth muscles at all times

ACKNOWLEDGMENT

The authors wish to thank Dr Z E. Zanetti of the Department of Organic Chemistry, for his generous contribution of the various furfuryl amines studied in this paper

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THE ACTION OF METHYLENE BLUE UPON CYANIDE POISONING IN THE TURTLE[1]

J M. JOHLIN

From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, and Marine Biological Laboratory, Woods Hole

Received for publication November 29, 1940

It was shown by Johlin and Moreland (1) that the turtle can survive under conditions of complete anoxia for 30 hours or longer, acquiring a fermentable blood sugar concentration as high as 1100 mgm per cent and a blood lactic acid content as high as 1000 mgm per cent Moreland (2) showed that the histotoxic anoxia of cyanide poisoning produces almost equally high levels in the fermentable sugar and lactate content of the blood of the turtle even when the dosage is small enough to permit the animal to survive He also showed that the glycogen of the ventricles disappears almost completely during this anoxia, while considerable stores of glycogen remain in the muscles and liver

The present paper contains the results of an investigation of the oxygen consumption of turtles poisoned by cyanide, of the effect of methylene blue upon the oxygen consumption of poisoned animals, of the effect which methylene blue has upon the restoration of the heart glycogen and of its effect upon the recovery of cyanide from the respiratory gases It will be shown that methylene blue helps to maintain the bodily vigor of turtles poisoned by cyanide and to restore the heart glycogen, that it increases the oxygen consumption without immediately greatly lowering the high concentration of glucose and lactates in the blood, and that it greatly reduces the amount of cyanide to be recovered from the respiratory gases The data obtained apparently conform with Wendel's (4) postulation that the action of methylene blue is that of converting hemoglobin into methemoglobin in the presence of cyanides According to Wendel's view the addition of both cyanide and methylene blue to blood results in the rapid conversion of hemoglobin into methemoglobin The reduction of methemoglobin to hemoglobin, by leuco-methylene blue and the enzyme systems of the erythrocytes, which normally takes place when methylene blue alone is added, is prevented by the stabilizing action which cyanides have in the formation of the less easily reducible cyanmethemoglobin

[1] Aided by a grant from the Fluid Research Fund of the Rockefeller Foundation

MATERIALS AND METHODS

Practically all of the turtles used were the painted variety weighing between 500 and 700 grams. All analyses for glycogen for blood sugar and for lactate were made as described in two previous papers (1 2)

The rates at which HCN and carbon dioxide were expired and the rate at which oxygen was consumed were determined simultaneously The respiratory chamber was a glass desiccator having an approximate volume of 2000 cc carefully calibrated and connected with various calibrated devices for absorbing CO_2 and HCN and for automatically keeping the gas pressure within the apparatus constant by admitting oxygen as fast as it was consumed. The absorbing device was such as to produce a continuous circulation of the gases within the apparatus through the absorbing chamber for CO_2 and the connecting tubes which contained a silver nitrate solution for the absorption of HCN A similar absorbing device has been described heretofore by the writer (3) The two cyanide absorption tubes were placed between the two outlets of the CO_2 absorption chamber and those of the desiccator so that the cyanide circulating with the air of the desiccator to the CO_2 absorption chamber was removed from the circulating gas before it reached the latter chamber The pressure within this respiratory apparatus was read on a mercury manometer by means of a microscope cathetometer The apparatus was used in a constant temperature room at 20°

All injections of animals receiving sodium cyanide and methylene blue were made intramuscularly Unless otherwise stated, the dosage for cyanide was 25 mgm. per kilogram of body weight and that of methylene blue was 40 mgm. (total) pH determinations were made with the glass electrode. As in previous experiments, blood samples were taken under mineral oil.

RESULTS

Animals which were injected with 25 mgm. of sodium cyanide per kilogram almost invariably died Those receiving injections of 40 mgm. of methylene blue after the injection of cyanide invariably survived. Even when the methylene blue was administered 24 to 36 hours after the administration of the cyanide recovery occurred in the animals which were then still alive The heart glycogen of 13 animals which received cyanide alone and which died was invariably found to be greatly depleted at the time of death, varying from 0 04 to 0 09 per cent (table 1, nos. 22–34) The time of survival of these animals varied from 18 to 144 hours. Occasionally an animal which had not received methylene blue recovered Two such animals which were sacrificed three weeks later were found to have heart glycogen contents of 6 0 and 7 9 per cent respectively The blood sugar blood lactate and blood pH of these two animals had returned to normal levels. The average heart glycogen of 4 normal animals was found by Moreland to be 5.60 per cent, varying between 4.5 and 5 9 per cent.

Animals receiving cyanide alone became almost lifeless soon after its injection Those which were injected with methylene blue immediately after receiving cyanide remained active Those which still survived and received methylene blue 24 to 36 hours after receiving cyanide and which had become practically lifeless, became active again within a few hours. Yet

methylene blue, even though it was injected immediately after the cyanide, did not prevent the heart glycogen from reaching a low level as may be seen

TABLE 1

Heart glycogen of turtles at various intervals following the injection of both methylene blue and sodium cyanide (1–19), and of turtles receiving cyanide only (19–34)

ANIMAL NUMBER	TIME INTERVAL BETWEEN CYANIDE AND METHYLENE BLUE INJECTIONS	TIME INTERVAL BETWEEN CYANIDE INJECTION AND GLYCOGEN DETERMINATION	PER CENT HEART GLYCOGEN
1	None	18 hours	0 19
2	None	18 hours	0 07
3	None	24 hours	0 09
4	None	24 hours	0 04
5	None	36 hours	0 58
6	None	36 hours	0 38
7	None	36 hours	0 41
8	None	84 hours	1 71
9	None	144 hours	1 71
10	None	3 weeks	5 60
11	None	3 weeks	4 83
12	None	3 weeks	4 54
13	24 hours	120 hours	1 28
14	24 hours	120 hours	1 39
15	24 hours	120 hours	1 34
16	32 hours	165 hours	1 65
17	36 hours	130 hours	0 89
18	36 hours	165 hours	1 00
19	Received cyanide only	18 hours (S)	0 07
20	Received cyanide only	24 hours (S)	0 06
21	Received cyanide only	36 hours (S)	0 05
22	Received cyanide only	18 hours (D)	0 07
23	Received cyanide only	24 hours (D)	0 07
24	Received cyanide only	24 hours (D)	0 06
25	Received cyanide only	24 hours (D)	0 04
26	Received cyanide only	24 hours (D)	0 09
27	Received cyanide only	26 hours (D)	0 04
28	Received cyanide only	32 hours (D)	0 09
29	Received cyanide only	32 hours (D)	0 09
30	Received cyanide only	36 hours (D)	0 05
31	Received cyanide only	72 hours (D)	0 06
32	Received cyanide only	80 hours (D)	0 04
33	Received cyanide only	96 hours (D)	0 06
34	Received cyanide only	144 hours (D)	0 09

(S) Animal was sacrificed at time indicated
(D) Animal was killed at time indicated

by the data of table 1 The administration of methylene blue appears, however, to have effected a rapid recovery of the heart glycogen Animals 1 and 2 which were sacrificed 18 hours after receiving cyanide and methylene

blue were found to have heart glycogens of 0 19 and 0 07 per cent Animals 3 and 4, which were sacrificed after 24 hours, also had almost exhausted their stores of heart glycogen Animals 5–12, which were sacrificed at progressively later periods, had a progressively increasing amount of heart glycogen which was found to have returned to a normal state within 3 weeks Animals 13–18, which survived cyanide poisoning for 24 and 36 hours and then received methylene blue soon recovered their bodily vigor and increased their heart glycogen (assuming that the heart glycogen had reached as low a level as that of animals 1–4)

Moreland found the average pH of the blood of animals receiving 100 mgm. of cyanide to be 6 75, 20 hours later while in animals receiving only 20 mgm. the pH was found to be 7.38 The blood bicarbonate of animals receiving 20 mgm. of sodium cyanide was as low as that of animals receiving 100 mgm. of the poison. Apparently the animals receiving the lesser amount of cyanide had retained the ability to overventilate sufficiently to maintain an almost normal blood pH

In the present investigation it was found that the blood pH of nearly all animals receiving both methylene blue and cyanide was near an average of 7.52 at the time when they were sacrificed. That of one animal, sacrificed 36 hours after injection, was 7 75 (The average normal pH of 15 animals was found by Moreland to be 7 75)

The retention of their bodily vigor by those animals which received methylene blue after injections of cyanide does not signify the absence of a relatively high level of glucose and lactates in the blood The animal which, at 36 hours after injection had a blood pH of 7 75 also had a blood lactate content of 432 mgm. per cent and a blood sugar of 204 mgm per cent This condition might be brought about by hyperventilation In the case of another animal sacrificed 83 hours after injection the blood pH was 7 57 and the blood glucose and lactate 727 and 308 mgm. per cent respectively The severe acidosis which is to be found in animals about to die of cyanide poisoning is due not only to a depleted alkali reserve, but to respiratory failure when the animal has become completely exhausted

One animal received 25 mgm. of sodium nitrite per kilogram of body weight following the injection of cyanide It was killed 168 hours later having retained its vigor during this period. At this time it had a blood pH of 7 41, glucose and lactate contents of 286 and 114 mgm. per cent and a heart glycogen of 1 65 per cent

Preliminary to determining the effect which cyanide has upon the oxygen consumption of turtles it was found that 2 normal animals had respiratory quotients of 6.8 and 7 0 and that their hourly oxygen consumptions were 87 0 cc and 21.5 cc

To determine the effect which cyanide both in the presence and absence of methylene blue has upon the oxygen utilization the respiratory quotient

and hourly oxygen consumption, were determined on the basis of the oxygen consumed and the CO_2 expired during consecutive periods of 2, 4, 6, 12 and 24 hours following the injection of cyanide or of cyanide and methylene blue Some animals were injected with 25 mgm of NaCN per kilogram and others

TABLE 2

(a) Respiratory quotient, (b) hourly oxygen consumption cc , (c) per cent HCN recovered from respiratory gases, in successive periods

Number of experiment	Mgm NaCN per kgm body weight	Mgm methylene blue	Initial 2 hours	From 2nd through 8th hour	From 8th through 12th hour	From 12th through 24th hour	From 24th through 48th hour	From 48th through 72nd hour	From 72nd through 96th hour	From 96th through 120th hour	From 120th through 144th hour	From 144th through 168th hour	From 168th through 192nd hour	Total recovery of HCN	Remarks
1a	25	—	15 8	5 3	—	3 2	3 0	1 13							Died
2a	25	—	10 2	5 3	3 7	2 3	0 80	0 62	0 53	1 13					Died
3a	75	—	13 4	8 8	6 6	17 3	11 7	2 7	6 8						Died
4a	75	—	18 0	6 8	4 3	4 5	1 9	1 3	1 0	1 25	0 88	0 81	0 85		Died
5a	25	40	11 2	3 0	2 0	1 5	1 1	0 75	0 48	0 43	0 45				Survived
6a	25	40	4 0	1 7	1 2	0 91	0 61	0 47	0 57	0 56	0 44				Survived
7a	25	40	5 6	1 8	1 2	0 97	0 84	0 57	0 53	0 60	0 65	0 66	0 63		Survived
8a	75	40	13 8	9 4	8 0	5 0	2 7	1 6	0 73	0 37					Died
9a	75	40	8 2	—	6 6	3 0	1 3	0 62	0 52	—					Died
1b	25	—	4 6	3 6	—	5 0	4 3	9 5							
2b	25	—	7 7	5 5	4 8	6 0	11 6	18 9	13 5	1 7					
3b	75	—	3 9	2 1	3 4	0 8	0 9	2 4	0 7						
4b	75	—	3 4	2 5	3 8	3 4	5 8	6 6	4 4	4 4	4 1	4 4	5 2		
5b	25	40	8 2	11 3	10 6	9 2	11 6	17 7	20 8	24 0	21 2				
6b	25	40	16 8	21 4	21 0	20 9	26 1	28 1	28 6	32 3	28 3				
7b	25	40	11 3	16 4	16 2	15 3	17 7	24 7	26 0	23 8	22 3	23 8	24 2		
8b	75	40	2 8	3 3	3 3	3 0	3 5	4 4	4 8	4 6					
9b	75	40	4 8	—	3 3	4 4	4 2	5 4	7 0						
1c	25	—	2 5	2 8	3 2	4 7	6 9	2 4						22 5	
2c	25	—	2 3	1 8	2 1	1 8	3 8	2 2	2 1	2 6				18 7	
3c	75	—	5 3	4 7	4 7	6 4	9 5	4 9						35 5	
4c	75	—	6 7	4 5	6 1	5 5	7 2	4 3	2 8	3 3	1 1	1 1	0 0	42 6	
5c	25	40	2 2	→	→	→	1 4	→	0 6	0 8				5 0	
6c	25	40	1 8	→	→	→	1 8	→	0 3					3 9	
7c	25	40	0 8	→	→	→	→	→	→	→	→	1 1		1 9	
8c	75	40	5 1	2 4	1 1	1 2	3 1	3 4	1 2	1 2				18 7	
9c	75	40	→	→	→	→	→	→	14 9					14 9	

with 75 mgm Some were given simultaneous injections of 40 mgm of methylene blue The data thus obtained are given in table 2 The respiratory quotient is at first very high whether or not the animal had received injections of methylene blue immediately after those of cyanide This may be attributed both to hyperventilation and to the formation of lactic acid When methylene blue was not administered the respiratory quotient gradually

decreased in value from that of the first two hours, but usually remained above the normal until the animal died. When the animal received an injection of methylene blue immediately after that of 25 mgm. of cyanide, the respiratory quotient continued to decrease from its initial high value more rapidly and eventually fell considerably below the normal. This is no doubt due to the disappearance of lactates and the restoration of the bicarbonates of the alkali reserve. As was demonstrated by Johlin and Moreland the carbonates lost during anoxic anoxia, contributing to the pronounced acidosis, equal an amount greater than those contained in all of the body fluids of the animal and are, therefore lost from the bony structure as well.

The hourly oxygen consumption of animals which had received methylene blue as well as cyanide was, in general, considerably greater than that of those which received only cyanide.

The amount of cyanide recoverable from the respiratory gases was determined simultaneously with the respiratory quotient and the hourly oxygen consumption. The percent recovery of cyanide from the respiratory gases of animals which had received 75 mgm. of cyanide per kilogram of body weight was much greater than that from those which received but 25 mgm. The recovery of cyanide was greatly diminished by the injection of methylene blue.

DISCUSSION

Of the existing points of view regarding the action of methylene blue upon cyanide poisoning the present data would appear to support Wendel's claim (4) that the stabilizing influence of cyanides upon methemoglobin favors the oxidation of hemoglobin to methemoglobin by methylene blue and this brings about the withdrawal of cyanides from the cytochrome system of the respiratory mechanism to re-establish the activity of the latter system. The cyanide recoveries, as illustrated by the data of table 2, are much greater in the absence of methylene blue in the animal body than in its presence. Methylene blue greatly reduces the amount of cyanide recovered when the dose is large as well as when the approximately minimal lethal dose of 25 mgm. per kilogram of body weight is administered. The hourly oxygen consumption, induced by methylene blue, on the other hand is much less when the amount of cyanide is 75 mgm. than when it is 25 mgm. If methylene blue temporarily replaced the cytochrome system, then it would seem that this difference should not be so great.

The hourly oxygen consumption of animals which are injected with 25 mgm. of cyanide and 40 mgm. of methylene blue is well within normal limits within a few hours after their injection. That the metabolism of such animals is nevertheless somewhat extraordinary is illustrated by the glucose and lactate content of their blood which in one instance was found to be 727 and 308 mgm. per cent respectively 83 hours after the injection when the animal seemed to be enjoying its full vigor These figures for glucose and

lactate are comparable with those obtained when an animal is injected with cyanide alone As pointed out by Wendel, the normal functions of animals which have been poisoned by cyanide are by no means completely restored to their normal state by methylene blue It would appear that the pathway of an oxidative recovery following the administration of cyanide remains blocked and that the hepatic regulation of the blood sugar and the deposition of glycogen is disturbed even when methylene blue is administered It cannot be stated that more than one metabolic path involving different chemical trends is involved Similar complicated situations involving lactates but not glucose have been reported Ehrenfest and Ronzoni (5) found an accumulation of lactates in frog muscle under aerobic conditions after it had been treated with dinitrophenol Garrey and Boykin (6, 7, 8) found that the inhibition of the turtle's auricle is accompanied by a reduction of its oxygen consumption amounting to as much as 84 per cent of the resting oxygen utilization and that this reduction is not an essential feature of the inhibitory process They found the oxygen to be reduced 68 per cent by cyanide poisoning which, however, does not interfere with inhibitory processes They suggest that "cellular oxidation may be the resultant of two different chemical trends"

This investigation was carried on with the technical assistance of David Stewart and Sam Cecil

CONCLUSIONS

The heart glycogen of turtles receiving injections of methylene blue following those of sodium cyanide does not remain depleted as it does when they receive cyanide alone The glucose and lactate contents of the blood, however, remain at high levels long after the administration of the methylene blue or of sodium nitrite

The oxygen consumption of turtles poisoned by cyanide is very considerable but is greatly increased and brought within the limits of normal levels by methylene blue

The amount of cyanide recoverable from the respiratory gases of turtles poisoned by cyanide is greatly decreased by methylene blue

These facts would appear to support the theory that methylene blue converts hemoglobin into methemoglobin under the stabilizing influence of cyanides rather than that it takes the place of the poisoned respiratory enzymes of the cytochrome system of the respiratory mechanism

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THE RELIABILITY OF THE COBALT ISOPROPYLAMINE COLOR REACTION FOR AMYTAL

THE EVALUATION OF CHROMOGENIC SUBSTANCES IN URINE[1]

R. F KRAUSE AND RICHARD F RILEY

From the Department of Biochemistry and Pharmacology School of Medicine and Dentistry The University of Rochester Rochester New York

Received for publication December 3 1940

Koppanyi and Krop (1) using a cobalt colorimetric method have reported an average of 8 per cent of administered amytal excreted in the urine. This is at sharp variance with the reports of Herwick (2) and Shonle *et al.* (3) that no amytal could be recovered by sublimation from such urine. Since the colorimetric method has recently been shown to lack the specificity originally ascribed to it (4) and since this method has been generally used for barbiturates it is important to establish its reliability quantitatively As is shown below not only has the precision of the method been improved, but an explanation of the disparity between the previous findings on amytal excretion has been made.

RELIABILITY OF THE COLORIMETRIC METHOD

The method of Koppanyi *et al.* (5) for amytal gave an average variation in color intensity of 7 per cent in duplicate determinations on standard amytal solutions whose concentrations ranged from 18–300 mgm. per cent. Kozelka *et al.* (6) previously showed that when fixed amounts of cobalt and base are used for different quantities of the barbiturate, the color intensities were not proportional to the amount of the barbiturate present. These errors are now shown to be due largely to the use of sub-optimal amounts of reagents (figs 1 and 2) These curves were determined from solutions of amytal in chloroform and were not urine extracts (*v.i.*) By a suitable choice of amounts and concentrations of the reagents, the average error of duplicate determinations was reduced to 1.5 per cent for the concentration range 18–300 mgm. per cent.

These results indicate that definite amounts of isopropylamine and Co^{++} are necessary for the formation of an optimum color intensity This is in agreement with the results of Kozelka and Tatum (7) who employed sodium

[1] This work was supported in part by a grant from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

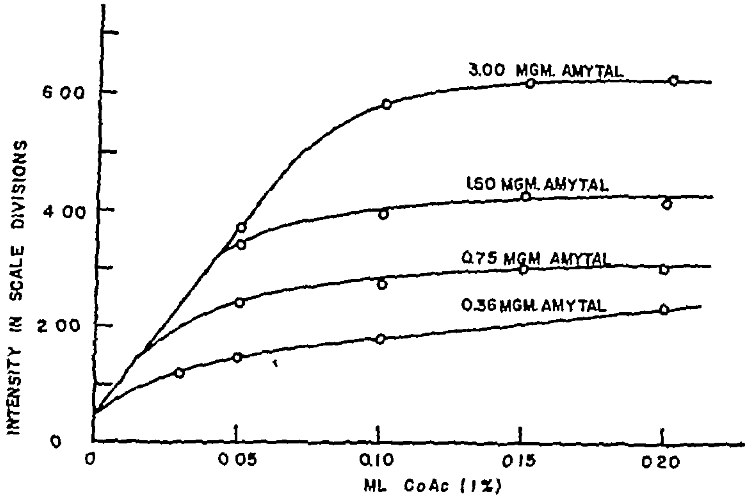

Fig 1 Variation of Color Intensity at Different Cobaltous Acetate Concentrations (0 3 *cc* of a 5 Per Cent Solution Isopropylamine)

Abscissa, cc of 1 per cent cobaltous acetate in absolute methyl alcohol Ordinate, color intensity in arbitrary scale divisions Amounts of amytal indicated, dissolved in 3 0 cc of chloroform, were used in each determination on their respective curves No maximum was observed for 0 36 mgm of amytal, due to the relatively greater increase in cobalt color in comparison with reaction color

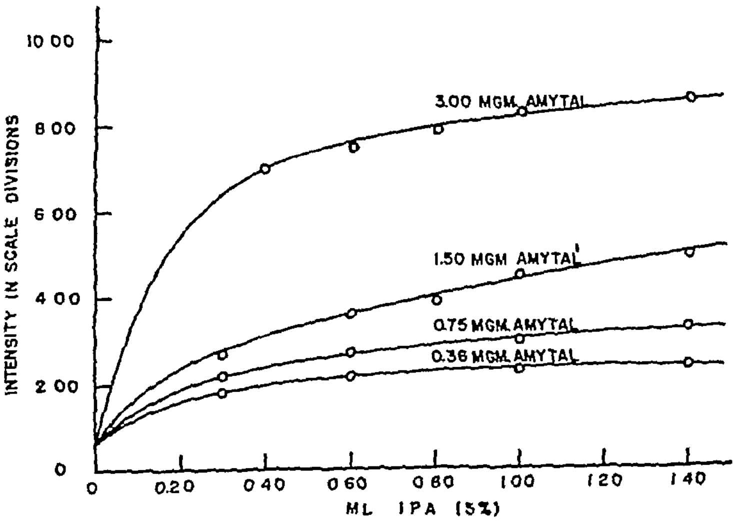

Fig 2 Variation of Color Intensity at Different Isopropylamine Concentrations (0 2 cc of a 1 Per Cent Solution Cobaltous Acetate)

Abscissa, cc of 5 per cent isopropylamine in absolute methyl alcohol Ordinate in arbitrary scale divisions Amounts of amytal indicated, dissolved in 3 0 cc of chloroform, were used in each determination on their respective curves

ethylate as a base. Comparison of the curves in figures 1 and 2 with those of Kozelka and Tatum shows some differences. Their curves break sharply at the maximum while those shown above approach maxima slowly The differences may be due to greater dissociation in our solutions which were more dilute On the basis of concentration per cc of total volumes, 5–38 times less amytal was employed, while about the same abount of Co^{++} but 10 times the amount of base were used Thus, more dilute solutions of the colored substance were produced

Method of preparation of extracts and development of color

Urine extracts were prepared according to the following method Fifty cc samples of urine to which had been added 10 cc of 1 0 *N* sulfuric acid were extracted for ½ hour with 500 cc. of chloroform. The chloroform extracts were dried over anhydrous sodium sulfate reduced to a convenient volume (25–50 cc) by distillation and made up in 50 or 100 cc volumetric flasks.[1]

The following solutions were employed

1 1 per cent solution of cobaltous acetate (Baker's C.P) in absolute methyl alcohol

2 5 per cent solution of isopropylamine (E K Co) in absolute methyl alcohol.

In developing the color the following procedure[2] was employed To 0.2 cc of 1 per cent cobaltous acetate, in a colorimeter cup was added, in the following order 0.6 cc of 5 per cent isopropylamine, 2.0 cc of chloroform and 1 0 cc of the chloroform extract. The solutions were well mixed by rotation, and the intensity of the color produced read on a photoelectric colorimeter

The use of a color filter was considered. However it has been shown (4) that the cobaltous acetate solution has a maximum color absorption at 5150 Å, cobaltous acetate and isopropylamine at 5450 Å and amytal plus these reagents at 5650 Å. Any filter which would remove the 5150 Å or the 5450 Å bands would also remove so much light from the 5650 Å band that the efficiency of the method would be seriously impaired. Consequently no color filter was used

Urine pigments and chromogenic substances

Two sources of additional colors have been identified and attempts made to correct for each. These sources are (*a*) urine pigments extracted by chloroform and (*b*) chromogenic substances, i.e non-barbiturate substances in the chloroform extracts of urine which react with the cobaltous acetate reagents to produce color

Determination of extract color To evaluate the color due to urine pigments, a 1 cc aliquot was added to 2.8 cc of 20 per cent methyl alcohol in chloroform in a colorimeter cup These were well mixed and the color intensity of the extract was read on the photoelectric colorimeter The use of 2.8 cc of 20 per cent methyl alcohol in chloroform duplicates the conditions of solvent composition and total volume employed in the color reaction. The color of the extract may represent an apparent excretion of a considerable amount of amytal e.g 10 mgm per 100 cc of urine (table 1)

Treatment with acidic copper sulfate has been recommended prior to chloroform extraction This treatment had no appreciable effect on extraction color (table 1) or in preventing emulsification. Treatment of the dry chloroform extracts with Norite removes much of these pigments however in extracts containing amytal varying

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[1] Treatment with anhydrous sodium sulfate was shown to remove no detectable amount of amytal from known chloroform solutions.

[2] Koppanyi's (5) procedure used 0 05 cc of cobaltous acetate solution and 0.3 cc of the isopropylamine

amounts of the barbiturates are also removed Levvy (8) and Brundage and Gruber (9) have shown a similar adsorption behavior for other barbiturates

Determination of chromogenic substances To evaluate the color produced by non barbiturate substances in the chloroform extracts, normal urine of dogs receiving no amytal was extracted and the color developed by the above methods In table 1 are

TABLE 1

Color reaction in urine extracts Dogs received no amytal

	Untreated			$CuSO_4$ treated		
	Color intensities (scale divisions)		Amytal equivalent	Color intensities (scale divisions)		Amytal equivalent
	Average	Range		Average	Range	
			mgm / 100 cc.			*mgm / 100 cc.*
Total color extracts plus reagents*	0 20	0 09–0 37	21	0 19	0 05–0 48	20
Extract color	0 10	0 04–0 17	10	0 10	0 01–0 24	10
Color due to chromogenic substances (difference)	0 10		11	0 09		10

* The "total color" values have been corrected for the color due to the reagents, *per se*

TABLE 2

Color reaction in urine extracts Dog received amytal

Dosage	Number of determinations	Average color intensities* expressed as percentage of amytal administered	
		Recovered in first 24 hours	Recovered in second 24 hours
Oral administration			
mgm /kgm			
35	4	4	3
50	4	6	2
76	3	6	15
79	3	14	6
Intravenous administration			
42	3	13	11
50	3	6	10

* Corrected for extract color and color due to chromogenic substances

given the data on 12 such samples, each sample was divided and one-half treated with copper sulfate prior to extraction with chloroform As may be seen, the chromogenic substances in the urine extracts provide an apparent average value of 10 mg of amytal per 100 cc of urine The total color has an average equivalent of 20 mgm of amytal per 100 cc of urine Since our dogs usually excreted 500 cc of urine per day, these two

sources of color account for an apparent excretion of 100 mgm of amytal per day in a dog receiving no amytal

The extract color can easily be determined on urine samples after amytal administration however the color due to non-barbiturate chromogenic substances cannot be assayed An attempt to discover a mathematical correlation between the extract color and the color due to chromogenic substances only demonstrated a high degree of independent variability Therefore because of the wide range in both color intensities (table 1) rigorous corrections cannot be made However as a first approximation the extract color was determined and the color due to chromogenic substances taken as equal to this value

INCREASES IN COLOR INTENSITIES AFTER AMYTAL

Since the method error has been markedly reduced and since two sources of extraneous color have been identified and given a rough quantitative evaluation it seemed worth while to repeat Koppanyi and Krop s (1) colorimetric studies of urine from dogs given amytal In general, their data, on the basis of color only have been confirmed (table 2) significant increases in color intensities are obtained after amytal administration either orally or intravenously

DISCUSSION

Koppanyi and Krop (1) accepted the increase in color intensities as evidence of amytal excretion There are several reasons why this interpretation should be regarded as doubtful. (*a*) The color reaction is given by a number of non-nitrogenous substances (4) For instance isoamyl ethyl acetic acid, gram for gram, gives half as much color as amytal This is of major interest here since this compound has been named as a possible metabolic breakdown product of amytal (3) Other organic acids (e.g. acetic and butyric) also give the color reaction (4) (*b*) The adsorption maxima (4) of the colors produced by amytal (max. 5650 Å) and by other chromogenic substances (max. 5600–5750 Å) are so similar it is difficult to determine by this means the presence or absence of amytal in urine from dogs receiving the drug (*c*) No amytal precipitate was obtained when copper sulfate was added to a pyridine solution of the residue from the chloroform extract of urine of dogs receiving amytal (Zwikker test (10)) According to the color intensities measured, the combined chloroform extracts should have contained about 500 mgm of amytal. To check the sensitivity of this test, 10 mgm of amytal were added to normal urine and the Zwikker test repeated In this test, a precipitate appeared (*d*) Using the mouse injection test, Herwick (2) and Shonle *et al* (3) failed to recover any amytal by sublimation from similar solvent extracts. Because of the non-specificity of the color reaction and the negative chemical and physiological tests for amytal it would seem advisable to designate the increased color intensities observed as apparent amytal excretion until positive evidence establishes the presence of the drug

SUMMARY

1 The cobalt-isopropylamine color reaction for the determination of amytal has been studied critically Optimal amounts of reagents are recommended which reduce the method error to less than 2 per cent for concentrations of amytal of 20–300 mgm per cent

2 Dog urine normally contains pigments which are extracted by chloroform and which are included in the total color of the amytal reaction This factor has been accounted for

3 Dog urine normally also contains non-barbiturate chromogenic substances extracted by chloroform which produce color in the amytal reaction An attempt has been made to estimate this factor quantitatively

4 Dogs fed amytal excrete, in the urine, an increased amount of color producing substances This increase cannot be taken as proof of amytal excretion

Acknowledgements The authors wish to express their gratitude to H C Hodge for his helpful suggestions and criticism and to F E Hunter for technical assistance They also wish to thank Eli Lilly and Company, especially M M Weaver, for a generous supply of amytal and for a number of the proposed breakdown products of amytal

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THE EFFECT OF HISTAMINE ON THE PULMONARY BLOOD PRESSURE OF VARIOUS ANIMALS WITH AND WITHOUT ANESTHESIA

R. A. WOODBURY AND W F HAMILTON

From the Department of Physiology and Pharmacology University of Georgia School of Medicine Augusta, Georgia

Received for publication December 14 1940

The action of histamine upon the pulmonary circulation differs greatly in various species of animals (1 2 3) In the rabbit pulmonary constriction is the important factor in elevating the pulmonary arterial pressure (4) In the cat the picture is more complicated Though it is established that histamine usually increases the pulmonary pressure controversy exists as to the mechanism responsible for this effect. In the perfused lung venous flow is decreased, which Dale and Laidlow (5) and Osawa (6) attribute to constriction of the pulmonary vessels. The observation of Anderes and Cloetta (7) that histamine reduces the lung volume while increasing the pulmonary pressure argues for the importance of pulmonary constriction. Dixon and Hoyle (4), however report that histamine increases the lung volume, the venous outflow from the intact lung, and the pressure in the left auricle. They conclude that the rise in pulmonary pressure from small doses of histamine is principally the result of an increased output by the right heart. In the dog the picture appears even more confused Manwaring Monaco and Marino (8) report increased resistance to perfusion with diminished outflow from the lungs According to Franklin (2) Mautner and Pick report that histamine contracts the pulmonary artery However, Ruhl (9) states that in the heart lung preparation the increased pulmonary pressure results from a marked increase in coronary flow Dixon and Hoyle (4) report that any rise is very transient and that the secondary prolonged fall is the important change in the pulmonary pressure in dogs. They conclude that this fall is evidently the result of a limited venous return due to constriction of the hepatic veins Yet the systemic peripheral vasodilation from histamine would be expected to increase the blood flow through the peripheral vessels. This could well equal or exceed the amount of the decreased flow through the liver

Since deep anesthesia may even abolish the rise in pulmonary pressure in cats (McDowall (10)) it appeared worth while to study the effects of histamine upon the blood pressures of unanesthetized as well as anesthetized dogs. The studies were extended to include anesthetized cats and rabbits.

METHODS

Using the "hypodermic" manometer (11, 12) simultaneous pressure pulses were optically recorded from several blood vessels In some cases these included the aorta, the superior vena cava, the pulmonary artery and the pulmonary vein The manometers were connected to these vessels with either cannulae or hypodermic needles Rapid injections of histamine into different ones of these vessels allowed pressure changes to be recorded while the drug actions were more or less localized at various sites

Experiments upon Unanesthetized Dogs Several weeks or even months before recording the blood pressure of these animals, angiostomy cannulae (13) were inserted The preparation and application of these cannulae differ from the description previously given by the authors (14) They are now being made from coin silver tubing (diameter in inches $\frac{1}{4}$ outside, $\frac{3}{16}$ inside, and $\frac{3}{16}$ outside, $\frac{1}{16}$ inside) and silver strips ($\frac{1}{2}$ inch width and $\frac{1}{16}$ inch thick) The basal portion consists of one piece of the larger size tubing The basal flange is made by sawing along one end and then bending the prongs outwards (see fig 1) On the other end a clamp is constructed This clamp serves to fasten

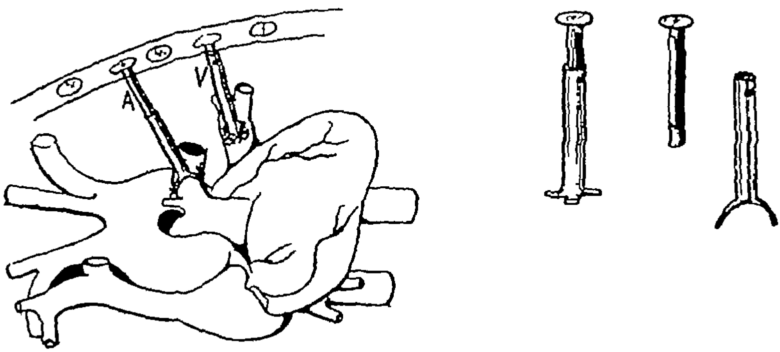

FIG 1 THE ANGIOSTOMY CANNULAE AND THEIR POSITION IN RESPECT TO THE CHEST WALL AND BLOOD VESSELS FROM THE HEART
A, pulmonary arterial cannula, *V*, pulmonary venous cannula

together the two parts of the cannula The second portion of the cannula is prepared from the smaller size tubing On one end a circular flange (made from a piece of the silver strip) is attached with silver solder This provides the flange which is placed just beneath the skin Along the middle section of this smaller-sized tubing, two surfaces are filed so as to make a beveled edge During the operation the clamp on the larger tube is closed onto these filed surfaces Then the two pieces of the cannula can not be drawn apart The telescope sliding joint is fitted loosely so that each heart beat will produce sliding of the cannula With the re-expansion of the lungs the cannulae will shorten themselves Trauma and stress at the attachments of the cannulae to the blood vessels are decreased

Using ether anesthesia these cannulae are applied to the pulmonary artery and vein and to the transverse arch of the aorta during a one stage operation Animals subjected to this type of operation (14) generally recover In several animals cannulae have remained in place upon the pulmonary vessels for over a year

Other modifications of the London cannula appear in the literature In place of the flange which is fastened to the vessel, the portal vein is encircled by a metal band

(Steigerwaldt, Reiser and Kurten (15)) and the pulmonary artery is encircled by a metal clasp (Katz and Steinitz (16)) The latter authors used cannulae that are adjustable in length, though the slide joint operates freely and the two portions can be pulled apart. In our hands the encirclement of the pulmonary artery with a metal hook or band proved unsatisfactory With this type of cannula two of the three operated animals recovered from the operation but these then died from the effects of hemorrhages on the 8th and 9th day Autopsy in both animals showed that the cannula had eroded through the pulmonary artery The period of survival of 9 and 11 days obtained by Katz and Steinitz (16) was about the same

From 5 to 10 mgm per kgm of morphine sulfate was injected subcutaneously into the animals one hour before each experimental study of the blood pressure. The animals would then stay quietly on the table during the experimental procedures The needles of the 'hypodermic manometers were inserted through the angiostomy cannulae and into the pulmonary artery pulmonary vein and aorta. In a few cases the jugular vein and femoral artery were exposed and needled under local anesthesia Before the histamine was injected strips of the record were developed and examined to make certain that the needles had satisfactorily entered their respective vessels

Experiments upon anesthetized animals Morphine sulfate and ether or pentobarbital-sodium alone were used for anesthesia Precautions were taken to minimize blood loss and trauma and to make the injections while the animals were in a light stage of anesthesia Cannulae were inserted into the pulmonary artery and vein in much the same manner as described by Johnson Hamilton, Katz and Weinstein (17) and the pressure pulses were recorded with hypodermic manometers.

RESULTS

Rabbits Injections of histamine phosphate increased the pulmonary pressure irrespective of whether it raised or lowered the systemic pressure Rapid injection of the drug into the jugular vein caused a rise in the pulmonary arterial pressure before any change occurred in the systemic pressure (fig 2 A) This means that histamine produces pulmonary vasoconstriction in rabbits anesthetized with morphine sulfate and ether

Cats The injection of histamine phosphate (0 01 to 0 5 mgm.) produced an elevation of the pulmonary arterial pressure in eight cats anesthetized with pentobarbital-sodium Four of these animals were in fair condition while the pressure pulses were recorded The contours of these records were similar to those obtained from dogs and gave values which averaged 33/18 mm Hg When histamine was injected into the superior vena cava the rise in the pulmonary pressure preceded the fall in the systemic pressure in two animals (fig 2 C) and accompanied it in the other two (fig 2 B) An increased blood flow may well contribute to the rise in the pulmonary pressure. However in the cat vasoconstriction of pulmonary vessels by histamine definitely does occur In some cats it is quite pronounced Even with small doses (0.5 mgm total) the pulmonary pressure gradient (pulmonary arterial pressure minus pulmonary venous pressure) during diastole becomes as high as 26 mm Hg in contrast to 12 mm Hg prior to the injection (fig 2 C) In this cat a larger dose of histamine actually raised the pulmonary systolic and diastolic pressures to values well above those simultaneously present in

the systemic vessels (fig 2 D) This was not the result of left heart failure because as shown in the figure the pulmonary venous pressure increased at first only 6 mm Hg and returned to its previous value in less than 15 seconds

When histamine (0 3 mgm) was injected into the aorta of one cat with very low (shock-level) blood pressures the reduction in the systemic pressure was only 8 or 10 mm Hg Blood flow may have decreased, certainly any

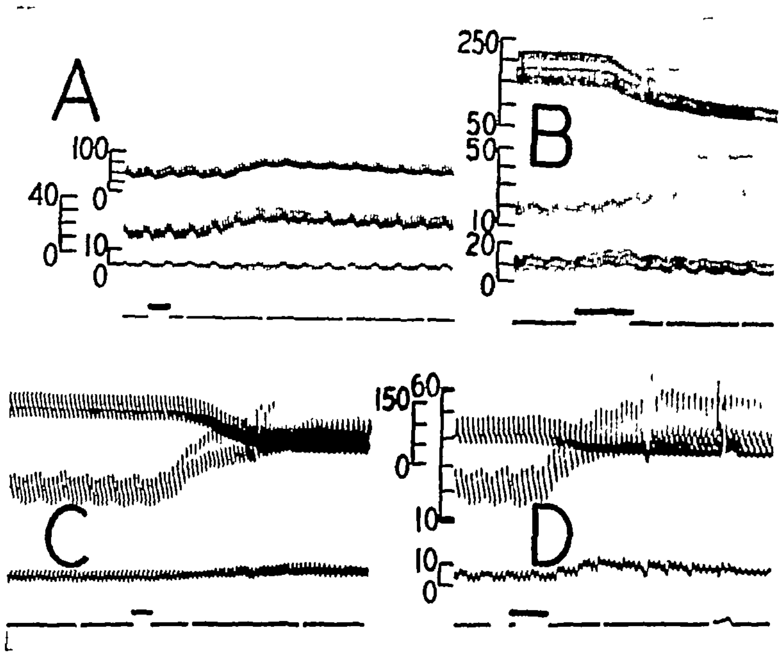

FIG 2 SIMULTANEOUS FEMORAL, PULMONARY ARTERIAL AND PULMONARY VENOUS PRESSURE PULSES (FROM ABOVE DOWNWARD IN EACH RECORD)

In all records the blood pressure scale indicates mm Hg and the base line is interrupted at intervals of 10 seconds *A*, rabbit anesthetized with morphine sulfate and ether At signal 0 2 mgm of histamine phosphate was injected into the jugular vein *B* and *C*, cats anesthetized with pentobarbital-sodium At signals 0 5 mgm of histamine phosphate was injected into the superior vena cava *D*, same cat as *C* At signal 2 mgm of histamine phosphate was injected into the superior vena cava

increase was insignificant However, a rise in the pulmonary pressure occurred It started twenty to thirty seconds after the fall in systemic pressure and preceded by three or four seconds the increased heart action which indicated the arrival of the drug at the coronaries (18)

Without doubt in anesthetized cats pulmonary vasoconstriction is an important factor in the rise of pulmonary arterial blood pressure from histamine In contrast to the results of Dixon and Hoyle (4), small as well as large doses produced evidence of pulmonary constriction in some of our cats

Dogs Intravascular injections of histamine phosphate (0 4 to 2 mgm /dog) were made into two *unanesthetized* dogs. Ten injections produced a rise in the pulmonary pressure without any secondary prolonged fall such as that observed in anesthetized dogs by Dixon and Hoyle (4)

Six rapid injections of the drug into the jugular or femoral veins of these dogs produced changes as shown in figure 3 A. The rise in the pulmonary pressure did not precede but followed the fall in the systemic pressure The pulmonary arterial pressure was not elevated during the first passage of the drug in relatively high concentrations through the lungs. Vasoconstriction in the pulmonary vessels, if it occurs is certainly ineffective and does not produce any rise in the pulmonary pressure

Four rapid injections into the arch of the aorta produced pressure pulse changes as illustrated in figure 3 B Immediately after the injection the systemic pressure decreased and the heart rate increased The simultaneous small fall in the pulmonary venous pressure indicates an increased left heart output associated with the more rapid heart rate and systemic vasodilation The rise in pulmonary pressure starts at least three seconds later and soon reaches its peak. This rise apparently results from an increased blood flow produced by peripheral systemic vasodilation and increased heart rate

The pulmonary pressure then returned to normal definitely before the heart rate and the systemic pressure (see fig 3 B) This very likely resulted from capillary pooling of blood (19) and possibly in part from constriction of the hepatic vein However any such limitation of the circulation of blood failed to reduce the pulmonary pressure below normal. In the absence of anesthesia and operative shock these animals possessed a definite reserve circulatory capacity and were able to make vascular adjustments.

In one of these dogs an injection of histamine was administered twenty *minutes after the intravenous injection of 30 units of 'Pitressin* *Among* other things the Pitressin had caused constriction of the coronaries and the systemic vessels and was markedly reducing the blood flow (20) Under these conditions the capacity to increase the blood flow after an injection of histamine would be expected to be diminished or abolished Even though the mechanism for pulmonary constriction should have been intact, histamine produced only a very small transient rise followed by a prolonged reduction in the pulmonary arterial pressure (fig 3 C) This indicates that the principal effects of histamine upon the pulmonary pressure result from changes in the blood flow The accelerated heart increased the left heart output and quickly lowered the pulmonary venous pressure Right and left heart output were then reduced The pulmonary arterial pressure pulses became mere ripples and the pulmonary pressures were reduced The large dose of Pitressin ' had injured the circulatory system Histamine was producing pooling of the blood in the capillaries (19) and the dog was evidently unable to make adequate vascular readjustments

In the four *anesthetized* dogs in which the pulmonary vessels had been

exposed and cannulated the pre-injection heart rates were more than double the normal values The responses to histamine varied, though following all

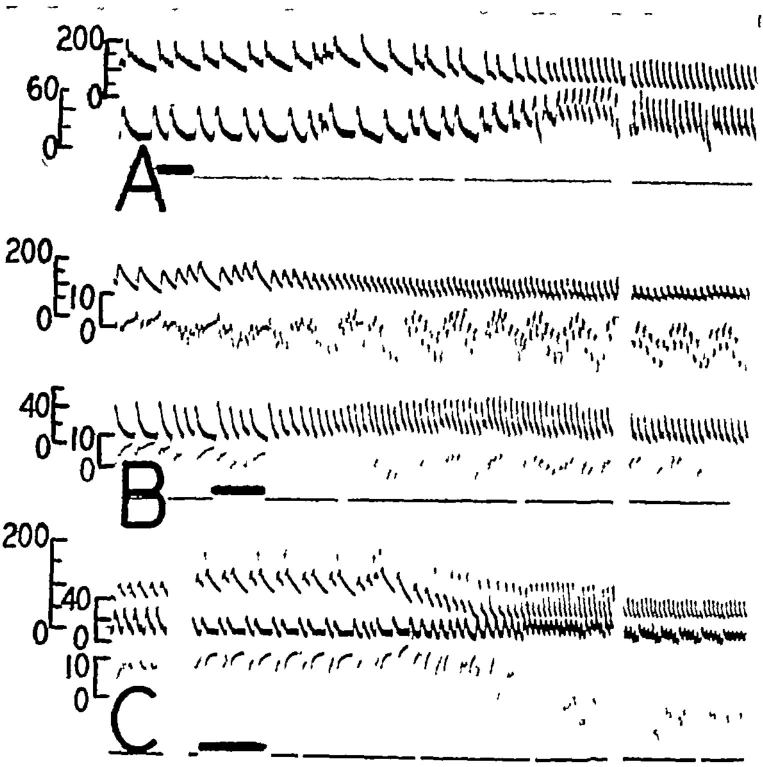

FIG 3 PRESSURE PULSES FROM THE SAME UNANESTHETIZED DOG (WEIGHT 14 KILOGRAMS) ON DIFFERENT DAYS

The break in each record at the extreme right indicates the lapse of 20 seconds *A*, simultaneous femoral and pulmonary arterial pressure pulses At the signal 2 mgm of histamine phosphate was injected into the femoral vein of the dog *B* simultaneous aortic, superior vena cava, pulmonary arterial and pulmonary venous pressure pulses (from above downwards) At the signal 0 5 mgm of histamine phosphate was injected into the aorta in the region of the arch *C*, simultaneous femoral pulmonary arterial and pulmonary venous pressure pulses At the left appear the normal pressure pulses During the first break in the record 30 units of "Pitressin" were injected intravenously and twenty minutes of the effects and recovery are deleted from this figure At signal, 0 3 mgm of histamine phosphate was injected into the femoral vein Compare with fig 3 A and 3 B which were taken from the same dog

injections the increases in heart rate and pulmonary pressure were small In two of these four dogs the transient small rise in pulmonary arterial pressure was followed by a prolonged fall, as was reported by Dixon and Hoyle (4)

Every injection of histamine (0 4 to 5 mgm.) caused this type of response in one dog after three hours of pentobarbital-sodium anesthesia (25 mgm. per kilogram) In the other animal the response changed from a small rise to the type with the prolonged fall after injecting an additional 5 mgm per kilogram of the barbiturate and waiting an hour (a total of 5 hours of anesthesia)

Very likely the explanation of the prolonged fall in the pulmonary pressure lies in the fact that prolonged operative trauma and the barbiturates (21) diminish the thoracic blood volume and blood flow Under these conditions histamine may fail to increase blood flow The capillary pooling of blood by histamine (19) may reduce blood flow to such an extent that the pulmonary pressure values are below normal Conditions which cause circulatory deterioration accentuate the secondary effect of histamine upon the pulmonary pressure

In dogs histamine affects the pulmonary blood pressure mainly through its influence on blood flow Pulmonary constriction possibly does occur However, it alone does not increase the pulmonary pressure as is the case in the anesthetized cat and rabbit.

SUMMARY AND CONCLUSIONS

Improved angiostomy cannulae have been attached to the aorta, pulmonary artery and pulmonary vein of dogs. The animals usually recovered and were still in good condition after six months Pressure pulses from these vessels were recorded while the dogs were unanesthetized

In dogs histamine phosphate intravenously raises the pulmonary arterial pressure. No evidence of effective pulmonary constriction was observed even during the first passage of the drug in high concentration through the lungs

Upon circulatory deterioration from any of several causes dogs ceased to respond to histamine by the sustained rise in pulmonary pressure. Instead the response was an initial transient rise followed by a prolonged fall. The capacity to increase blood flow determines the type of histamine response in dogs

In anesthetized cats and rabbits histamine produces effective vasoconstriction of the pulmonary vessels which increases the pulmonary arterial pressure

The pulmonary arterial pressure of four anesthetized cats averaged 33/18 mm Hg

Aid from The American Medical Association in carrying out this investigation is gratefully acknowledged

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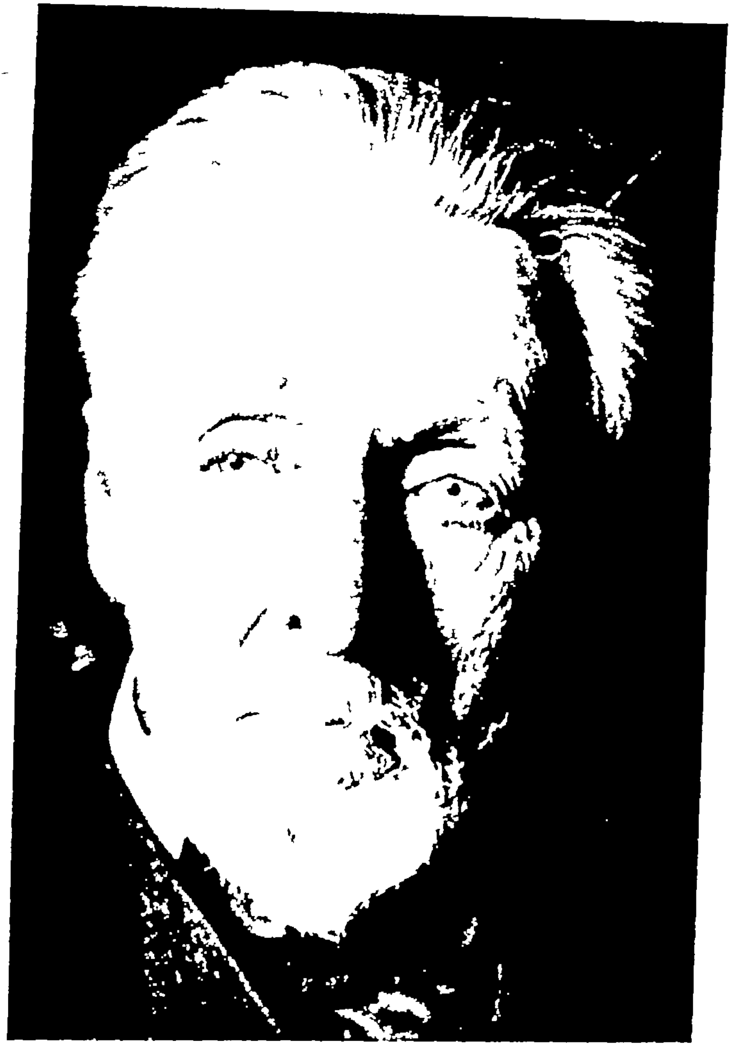

Hans Horst Meyer

Hans Horst Meyer

1853–1939

On October 6 1939, the American Pharmacological Society lost its oldest member when following a prolonged illness, Hans H Meyer Professor Emeritus of Pharmacology at the University of Vienna died in Vienna in his 87th year In his death the world lost one of the founders of modern pharmacology an eminent biologist, and a philosopher free from petty human failings.

Born on March 17 1853 in the East Prussian city of Insterburg he was the son of a prominent counselor of justice Dr Heinrich Meyer As a member of a well-to-do German family his early education was thorough and liberal Later at the gymnasium in Königsberg he received a more intensive training in the natural sciences with especial emphasis on chemistry and botany His medical studies, to which his innate talent and love for science led him were begun at the University of Leipzig where Carl Ludwig the famous physiologist and experimenter, made a profound impression on him. Then followed the study of clinical subjects at the University of Berlin where Traube and Frerichs were working Finally in 1877 he completed his doctorate thesis on the urea metabolism of chickens in Königsberg under Jaffe whose professorial chair combined physiological chemistry and pharmacology Jaffe offered Meyer a position as assistant but suggested that he go first to O Schmiedeberg at Strassburg to complete his training in pharmacological technique Schmiedeberg quickly recognized Meyer's talent and soon made him his assistant and co-worker

The years spent in Strassburg had a tremendous influence on Meyer's future career The work of this period has proved to be of lasting value including as it does the discovery of the camphor-glucuronic acid coupling, studies on the significance of alkalosis in respiration investigations of the action of phosphorous and iron and research on the Jaborandi alkaloids. It was during this period that Williams, with Meyer s guidance and substantial help devised his technique for the study of the isolated frog heart. Important as these years were for the development of Meyer's scientific genius, they were equally valuable for the young student because they offered opportunities for association with the men who came to Schmiedeberg s Institute from all parts of the world to study the then very young science of experimental pharmacology In these years Meyer began his lifelong friendships with the pharmacologists W von Schröder E. Harnack V Cervello A R. Cushny and F Williams

This fruitful period of work was interrupted in 1881 when Meyer, who had obtained the *venia legendi* at Strassburg University a short time before, was called at the age of 28 to succeed Boehm to the chair of Pharmacology at Dorpat, the only German University in the Russian Empire As he has said, the following years, during which he married his beloved wife Doris *née* Boehm, were the happiest of his life The social as well as scientific life of this small university town was very active and such men as Alexander Schmidt, G von Bunge, Draggendorf, A von Oettingen, and others who were then on the faculty, remained Meyer's steadfast friends throughout his lifetime A series of dissertations produced during this period included research on bismuth and aluminum poisoning, arsenic poisoning and acidosis

In 1884 Meyer was once again called to succeed Boehm, this time on the faculty of Marburg During the twenty year span in which he occupied the chair of pharmacology at this university he had the opportunity to conduct investigations into numerous problems of experimental therapeutics and pharmacology In this work he had the collaboration of native and foreign students among whom were Sobieranski, Eugen Rost, Otto Loewi, Walter Fletcher, C A Herter, F Ransom, V E Henderson, J T Halsey, W Hausmann, Fr Baum, and F Peters Out of the abundance of scientific work mention should be made of investigations of the influence of diuretics on the renal circulation, the rôle of tubular reabsorption, and the discovery of the fundamental importance of lipoid solubility for narcotic action It was during the Marburg period that Meyer studied diphtheria and tetanus toxins and made the important discovery that tetanus toxin spreads along the motor nerves to the spinal cord Other noteworthy investigations include those on the process of oxidation-reduction under the influence of methylene blue, on the effect of the corydalis alkaloids on the central nervous system and on the action of saponin on cholesterol of erythrocytes

In 1904 the Medical Faculty of the University of Vienna decided to create a Department of Experimental Pharmacology and asked Meyer to occupy the chair The new department soon attracted students from all parts of the world In addition to many Austrian and German co-workers, among whom were O Loewi, A Froehlich, Wiechowski, H Molitor, C Amsler, Fühner, Mansfeld, K. Cori and Knaffl-Lenz, there were numerous foreign research workers whose names were later to become famous, such as N Bruce, G H Whipple, G Barbour, P J Hanzlik, C Heymans, G Baehr, Airila, Ishizaka and others

Of the numerous studies relating to all branches of experimental pharmacology only a few will be mentioned here in order to indicate the wide field of work which Meyer managed to encompass He was the first to recognize the significance of Langley's theory of the functions of the vegetative nervous system to the explanation of the mode of action of pharmacological agents and he performed the first comparative experiments on the pharmacological

effects of adrenalin derivatives Among other contributions made by Meyer and his students during this time were the discovery of the antiphlogistic effect of local anesthetics experimental proof of the antiphlogistic action of calcium salts (due to their reduction of capillary permeability) demonstration of the central control of body temperature by means of local application of heat and cold to the *corpus striatum* investigation on the *transmission of* pain from the blood vessels through the sympathetic nervous system potentiation of the effect of adrenalin by cocaine, studies on the tonic innervation of striated muscle relaxation of bronchial spasm by adrenalin and investigation of the rôle of the liver as a reservoir for the regulation of the blood volume.

In addition to his experimental work, which carried the imprint of genius Meyer made a great contribution to pharmacology when in 1910 in collaboration with his friend R Gottlieb of Heidelberg he first published the textbook entitled 'Experimental Pharmacology as the Basis of Medical Treatment.' This textbook, which has appeared in nine subsequent editions was the first book to relate pharmacology as a biological science to the problems of rational therapy thereby establishing the importance of pharmacology in clinical teaching and medical practice Meyer believed that therapeutics should be based only on a sound experimental foundation. In the maintenance of this attitude, which foresaw the subsequent fruitful development of experimental pharmacology through a close alliance between experimental and clinical medicine Meyer had a brilliant prototype in his old clinical teacher and friend Bernhard Naunyn It was with this relationship in mind that in 1872 Naunyn with the pathologist Klebs and the pharmacologist Schmiedeberg founded the 'Archiv für experimentelle Pathologie und Pharmakologie which has served as a model for similar journals in other languages.

The importance which Meyer placed on the coöperation between pharmacologist and clinician is shown by the fact that shortly after the World War he and the cardiologist R Kaufmann established the 'Herzstation in Vienna where the results of experimental work could be applied at the sick bed to establish a rational therapy Another achievement equally characteristic of Meyer's practical mind was the creation of a government drug control bureau the purpose of which was not only to prevent the distribution of unsuitable drugs but also to encourage the production of rational therapeutic agents The legislation establishing this institution which Meyer directed until the last years of his life has served as a model for similar organizations in many other countries

Meyer's influence on his students was not confined to the professional side of their lives. It was always a great delight to come into contact with a man of such vast culture and truly profound wisdom A welcome opportunity for such contact was offered by the regular journal club meetings which were held in Meyer's hospitable home His excellent liberal and scientific education as well as his remarkable knowledge of the world's litera

ture made him an ever-stimulating speaker and gave him an inspiring and original philosophy of life.

Meyer was a paternal, solicitous and ever-willing friend. Not only his own students, but also many others whose abilities he recognized owed to him great advancement and in many cases the very foundation of their academic careers. At the same time he was a keen observer of human nature, endowed with a delightful sense of humor untouched by any trace of sarcasm, and with a rare appreciation of the good things of life. He had a legion of loyal admirers in all parts of the world with scarcely a personal enemy anywhere. The city of Vienna, which became his real home, bestowed honorary citizenship upon him, numerous native and foreign universities, academies and learned societies honored his scientific eminence. Those who had the good fortune of being close to him know how much kindness and love radiated from him and how his greatness was proved by his endurance of the pain and sorrow from which he was not spared. He will live forever as one of the great and noble men to whom science in general and pharmacology in particular is deeply indebted and to whom we and our successors shall always look up with admiration, gratitude and affection.

E. P. Pick

COOLING DRUGS AND COOLING CENTRES

F E. ROSENTHAL

From the Department of Experimental Medicine University College Leicester

Received for publication October 2 1940

This paper deals with the mode of action of certain drugs which lower body temperature in normal animals Harnack and his associates (11) first investigated the temperature lowering ('cooling') action of picrotoxin aconitine veratrine and santonin by injection into normal rabbits They suggested the possible existence of a temperature lowering (cooling') centre ('Kuehlzentrum) which might act by inhibiting peripheral metabolic processes. Meyer (19) supported this view and proposed further that the Kuehlzentrum' might act through the parasympathetic system He stressed that this group of "cooling' drugs as they may be called produced other signs of parasympathetic stimulation. Rosenthal and Licht (29a) found that these drugs do not lower body temperature in animals in which the cervical cord had been transsected. According to Licht (17) the cooling action of the drugs is accompanied by a decrease in oxygen consumption the depressing action on both body temperature and metabolic rate was said to disappear after cervical cord section According to Rosenthal Wallach and Friedländer (21a, b) the cooling action is diminished or annulled by hypnotics Experiments of Hashimoto (13) suggest that the mode of action of the antipyretic drugs differs from that of the cooling drugs It is relevant also to mention Cushing s (7) experiments showing that injection of pituitrin or pilocarpine into the cerebral ventricles produces signs of para sympathetic stimulation together with a fall of body temperature

The experiments to be described deal especially with the relation of the cooling drugs picrotoxin and aconitine to the vegetative centres in the hypothalamus

METHODS

In thirty two rabbits picrotoxin and aconitine were injected into the infundibular region the site of the heat regulating centres The skin of the head and the periosteum were anaesthetised with 1 cc of 0 5 per cent procaine hydrochloride solution A bilateral trephine opening was made in the skull at the junction of the coronal and sagittal suture 1.5 mm behind the coronal suture and 1 5 mm lateral to the sagittal suture A cannula of 0 4 mm diameter connected with a graduated insulin syringe was vertically inserted through these holes to a depth of 15 to 16 mm (Kym (16)) The amount of fluid injected on each side was 0 05 cc and after the injections the skin wound was

closed by stitches Aconitine in crystalline form was dissolved in normal saline with some granules of tartaric or acetic acid and then diluted as required The body temperature was taken rectally

RESULTS

Picrotoxin Bilateral para-infundibular injection of 0 01 mgm of picrotoxin gives rise to severe convulsions, usually followed by death 1 0 μ g

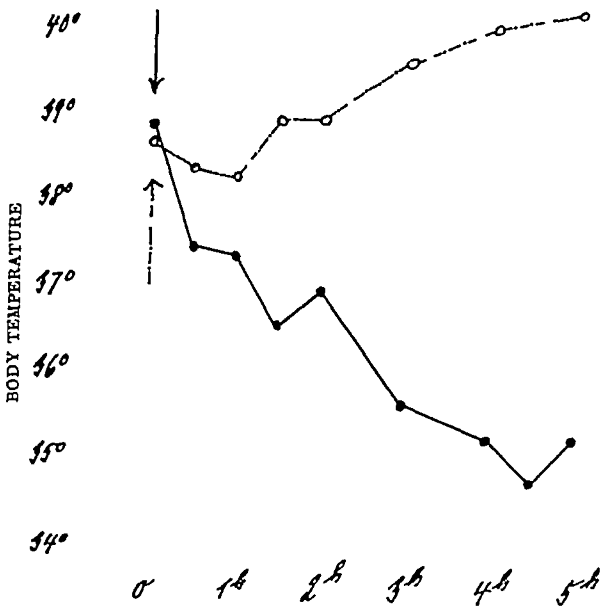

FIG 1 ⟶ Bilateral injection of 1 μg picrotoxin in 0 05 cc of distilled water into the infundibular region
--→ Bilateral injection of 0 05 cc of dist water into the infundibular region Control experiment
——— Decrease of temperature 4 1° C after infundibular injection of picrotoxin
— — — Increase of temperature 1 7°C after infundibular injection of dist water Effect of heat-puncture

($\frac{1}{1000}$ the effective subcutaneous dose) rarely produces convulsions but regularly lowers body temperature by about 2 to 4°C for 3 to 5 hours, accompanied by bradycardia Figure 1 gives an example of the temperature curve after bilateral infundibular injection of picrotoxin Injection of equal volumes of distilled water into the infundibular region, or the intracerebral injection of 1 0 μ g of picrotoxin into the frontal or occipital cortex is without temperature-lowering effect

Aconitine Still more striking cooling effects are obtained with this drug. Para-infundibular injection of as little as 0 03 μ g. ($\frac{1}{1300}$ of the minimal effective subcutaneous dose) may lower body-temperature by 5.3 to 5 6 C this effect may last for many hours and is accompanied as figure 2 demon strates, by hyperglycemia, the blood sugar rising from 95 to 230 mgm. per cent.

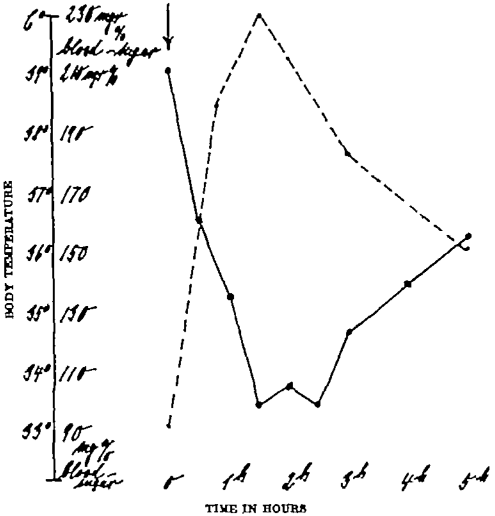

FIG 2 — Bilateral injection of 0 03 μg aconitine into the infundibular region
——— Decrease of temperature 5 6°C
— — — Increase of blood sugar from 95 to 230 mgm per cent.

Doses of aconitine of 0 015 to 0 03 μ g often, and 0 05 to 0 07 μ g. regularly give rise to cardiac changes. Electrocardiograms were taken by means of needle leads from (*1*) the right anterior limb to the lower part of the left side of the chest near the apex beat (*2*) from the spine to the sternum at heart level In experiments on 20 rabbits, para-infundibular injection of 0 015 to 0 03 μ g of aconitine produced bradycardia on thirteen occasions, and transient inversions of the T wave on nine Other changes noted after

larger doses of aconitine were nodal rhythm, extrasystoles of supra-ventricular and ventricular type, sino-auricular heart block, and para-arrhythmia, produced by the activity of two foci of about equal frequency The heart changes set in within 30 to 60 minutes of the injection, and passed off after two hours In some experiments, though the body temperature fell markedly, no cardiac changes occurred, so that the cooling action of the drugs is not simply the result of the changes in cardiac action

The cardiac changes were not produced by injection of 0 06 to 0 07 μ g of aconitine after bilateral vagal section They can therefore be attributed to central stimulation of the cardiac vagus

There is much collateral evidence that hypothalamic stimulation by electrical means or drugs or involvement in disease produces cardiac irregularities (1, 2, 3, 9, 15, 18) The disturbances of cardiac action produced by para-infundibular injection of aconitine are almost certainly due to stimulation of hypothalamic centres This suggests that the cooling effects of the drug are also the result of central stimulation rather than of central paralysis

Mode of action of picrotoxin The mode of action of picrotoxin can be investigated by considering the effect of hypnotic drugs on its cooling mechanism There is evidence that the remarkable antidotal properties of picrotoxin in barbiturate poisoning are caused by a reciprocal pharmacological mechanism Picrotoxin produces an awakening effect in barbiturate sleep, and barbiturates in hypnotic doses can protect against several times the lethal dose of picrotoxin (14, 22) The same applies with regard to the body temperature Our experiments show that the cooling effect of picrotoxin is suppressed by luminal and that picrotoxin annuls simultaneously the fall of temperature produced by luminal Figures 3 and 4 illustrate the results of two representative experiments In the experiment shown in figure 3, 0 05 g of luminal per kilogram body weight injected subcutaneously lower the temperature by nearly 5°C in 5 hours 1 2 mgm of picrotoxin per kilogram subcutaneously produced a maximum fall of temperature of 2 2°C after 2 hours Combined injection of luminal and picrotoxin subcutaneously produced a maximal fall of temperature of only 0 9°C at the end of an hour, the decrease of temperature is 1 3°C less than after picrotoxin injected alone

In the experiment shown in figure 4 the normal body temperature remains unchanged after the combined treatment with picrotoxin and luminal, although either luminal or picrotoxin alone lowers the body temperature

It may be suggested that picrotoxin, acting through undertermined channels, re-awakens the paralysed temperature centres to activity in the same way as it can re-awaken the activity of the cerebral cortex This would, of course, represent a different type of action in the narcotised animal from that produced by picrotoxin in the normal rabbit There are some analogies for a reversed action of substances under changed experimental conditions The reversal of the adrenaline effect after ergotoxin, the sympathetic action of

acetyl-choline in animals treated with sympathetic drugs (10) the stimulating action on the temperature centres and the awakening effect of atropine in hibernating animals (5) may be mentioned here

Paraldehyde acts similarly against the cooling effect of picrotoxin and picrotoxin *vice versa* against the hypothermia produced by paraldehyde (fig. 5) Although either paraldehyde or picrotoxin in this experiment produces

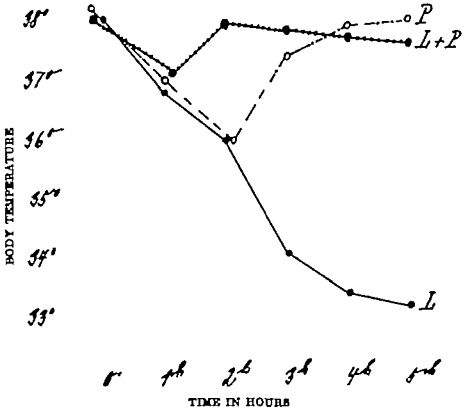

FIG 3 TEMPERATURE CURVES OF THE SAME RABBIT (TAKEN IN INTERVALS OF 5 DAYS)

——— 0 05 g luminal per kilogram subc (*L*)
— — — 1.2 mgm picrotoxin per kilogram subc. (*P*)
-·-·-· 0 05 g. luminal + 1 2 mgm. picrotoxin subc. (*L* + *P*)
Decrease of temperature after luminal (*L*) 4.8°C
Decrease of temperature after picrotoxin (*P*) 2.2°C
Decrease of temperature after luminal + picrotoxin (*L* + *P*) 0 9°C

a distinct lowering of body temperature normal temperature is maintained under the combined treatment with both substances

From these results it can be concluded (*1*) neither the picrotoxin-luminal nor the picrotoxin-paraldehyde antagonism is due to a direct chemical neutralisation but to a mutual pharmacological antagonistic action (*2*) As the cooling effect of picrotoxin can be suppressed by hypnotic drugs it cannot be due to paralysis of the temperature centres but must be due to stimulation of a centre (cooling centre) situated in the hypothalamus

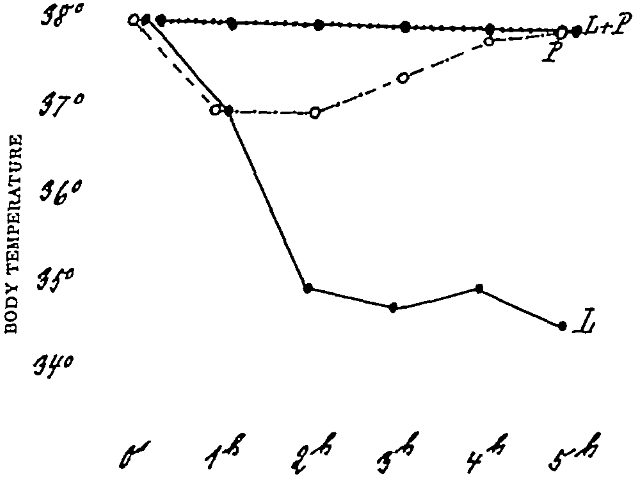

FIG 4 TEMPERATURE CURVES OF THE SAME RABBIT (TAKEN IN INTERVALS OF 5 DAYS)

——— 0 05 g luminal per kilogram subc (*L*)
— — — 1 3 mgm picrotoxin per kilogram subc (*P*)
-·-·-·- 0 05 g luminal + 1 3 mgm picrotoxin subc (*L* + *P*)
Decrease of temperature after luminal (*L*) 3 4°C
Decrease of temperature after picrotoxin (*P*) 1 0°C
Decrease of temperature after luminal + picrotoxin (*L* + *P*) 0

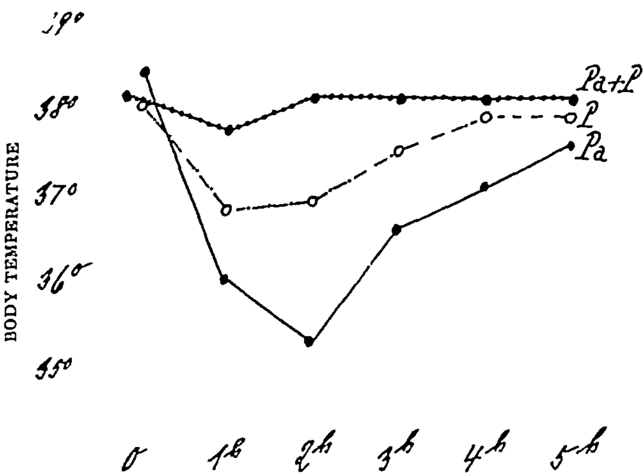

FIG 5 TEMPERATURE CURVES OF THE SAME RABBIT (TAKEN IN INTERVALS OF 5 DAYS)

——— 1 2 cc paraldehyde per kilogram, diluted with 30 cc of water, orally (*Pa*)
— — — 1 2 mgm picrotoxin per kilogram subc (*P*)
-·-·-·- 1 2 cc paraldehyde orally + 1 2 mgm picrotoxin subc (*Pa* + *P*)
Decrease in temperature after paraldehyde (*Pa*) 3 1°C
Decrease in temperature after picrotoxin (*P*) 1 2°C
Decrease in temperature after paraldehyde + picrotoxin (*Pa* + *P*) 0 4°C

The following experiments demonstrate that the cooling effect of picrotoxin is not suppressed in other kinds of experimental temperature fall

Relation of picrotoxin to temperature fall in sleep produced by calcium Injection of calcium chloride (0 1 to 0.25 mgm) into the region in the tuber cinereum in rabbits produces a condition resembling natural sleep which

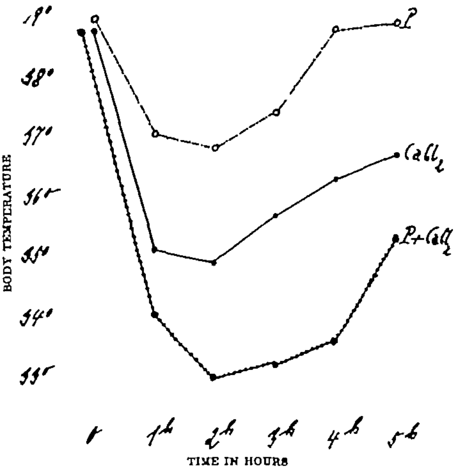

FIG 6 TEMPERATURE CURVES OF THE SAME RABBIT (TAKEN IN INTERVALS OF 5 DAYS)

——— 1 mgm $CaCl_2$ infundibularly injected on both sides (*$CaCl_2$*)
— — — 1.2 mgm picrotoxin per kilogram subc (*P*)
-·-·-·- 1 mgm CaCl infundibularly injected + 1 2 mgm. picrotoxin subc. (*$CaCl_2$ + P*)
Decrease of temperature after CaCl 3 8 C
Decrease of temperature after picrotoxin (*P*) 2 1°C
Decrease of temperature after $CaCl_2$ + picrotoxin (*$CaCl_2$ + P*) 5 6°C

lasts for ½ to 2 hours (6 8) there is also a fall of body temperature (12) Bilateral injection of 1 mgm of $CaCl_2$ prevents all forms of experimental fever (16) These effects are specific and cannot be produced by equimolecular solutions of other ions

Kym s technique was followed and 1 mgm of $CaCl_2$ dissolved in 0 05 cc of distilled water was injected into the infundibular region on both sides in

rabbits Figure 6 illustrates an experiment in which this procedure produced a maximum fall of temperature of 3 8°C after 2 hours 1 2 mgm of picrotoxin per kilogram injected simultaneously lowered the temperature by 2 1°C after 2 hours The combined injection gave a greater fall of temperature, 5 6°C after 2 hours This suggests that the temperature fall in the calcium sleep is produced by a paralysis of the temperature-raising (heating) centres, but the temperature lowering (cooling) centres stimulated by picrotoxin are intact Calcium sleep is antagonised by picrotoxin and the

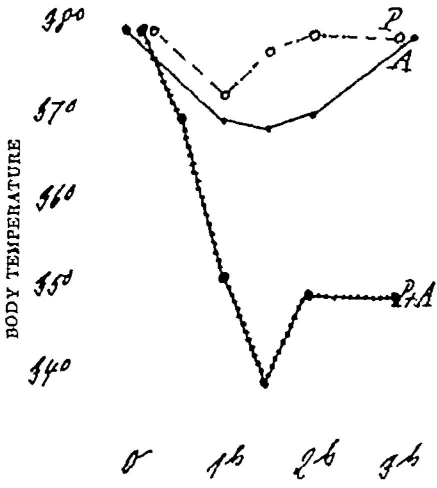

FIG 7 TEMPERATURE CURVES OF THE SAME RABBIT (TAKEN IN INTERVALS OF 5 DAYS)

——— 0 035 mgm aconitine per kilogram subc (A)
— — — 0 5 mgm picrotoxin per kilogram subc (P)
- - - - - 0 5 mgm picrotoxin + 0 035 aconitine subc (P + A)
Decrease of temperature after aconitine (A) 1 1°C
Decrease of temperature after picrotoxin (P) 0 7°C
Decrease of temperature after picrotoxin + aconitine (P + A) 4 0°C

animal wakes after ½ to 1 hour without any significant deviation in the temperature curve

Joint action of picrotoxin and aconitine on body temperature The cooling action of aconitine and picrotoxin simultaneously injected subcutaneously is far greater than the algebraic sum of the action of each This is well seen in figure 7 0 035 mgm of aconitine per kilogram produced a decrease of temperature of 1 1°C 0 5 mgm picrotoxin per kilogram lowered the temperature by 0 7°C The combined injection of both drugs gave a fall of temperature of 4 0°C after 1½ hours If 0 07 mgm of picrotoxin is combined with 0 04 to 0 06 mgm of aconitine per kilogram, there is marked bradycardia, inhibition of respiration, and finally death This potentiated effect combined

with signs of marked parasympathetic stimulation suggests that aconitine stimulates the same (cooling) centres as picrotoxin

DISCUSSION

The evidence presented above suggests that picrotoxin and aconitine stimulate a cooling centre in the diencephalon which is closely related to the centres controlling the parasympathetic system Collateral evidence is offered by other experimental observations that there are temperature raising (heating) and temperature lowering (cooling) centres within the general central temperature regulating mechanism Bruman (5) found that either a rise or fall of temperature can be produced in rabbits and cats by electric stimulation of various parts of the hypothalamus. Ransom, Fisher and Ingram (20) found that lesions of the hypothalamus produced a fall of temperature when the injury was placed behind the corpora mamillaria and a rise of temperature when the lesion was laterally placed in the rostral part of the hypothalamus.

The complex nature of the temperature fall produced by drugs is illustrated by the observations that picrotoxin annuls the fall of temperature by luminal or paraldehyde (and vice versa), but commonly has an additative action on the temperature fall during calcium narcosis and even a potentiating effect on the cooling mechanism of aconitine.

SUMMARY

1 Injection of picrotoxin (1 μ g) and aconitine (0 01 to 0 07 μ g) into the infundibular region produce in rabbits a deep fall of temperature accompanied by cardiac symptoms due to a central vagal stimulation

2 The cooling effect of picrotoxin is suppressed by luminal and paraldehyde but picrotoxin annuls simultaneously the fall of temperature produced by these drugs. In other kinds of experimental temperature fall (calcium sleep aconitine) picrotoxin shows an additative or even potentiated cooling effect.

3 The evidence so far collected in the experiments speaks in favour of the existence of temperature lowering (cooling) centres and supports the view of the dual structure of the temperature regulating centres

I wish to thank Mr F L. Attenborough M.A. Principal of the University College Leicester for the hospitality afforded me at the laboratories of the College I am indebted to Miss A Hosker B.Sc Ph D for the help given in carrying out the experiments and Prof S Wright for his revision of the manuscript.

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CLEARANCE OF THE DIETHYLAMINE AND DIETHANOLAMINE SALTS OF 3 5 DIIODO-4-PYRIDONE-N ACETIC ACID

D A. K BLACK J F POWELL, AND F H KEMP

From the Radcliffe Infirmary Oxford

Received for publication October 18, 1940

Ordinary Perabrodil or Pyelodil is a 35 per cent solution of the diethanolamine salt of *3 5* diiodo-*4* pyridone-*N*-acetic acid but in order to obtain more concentrated solutions of organic iodine for special purposes mixtures of Perabrodil with the diethylamine salt of the same acid have been used. Since the iodine-substituted pyridone in these two salts is the same it might be anticipated on chemical grounds that they would differ but little in their rate of excretion by the kidneys but it was felt that this should be confirmed by actual experiment Two normal subjects were therefore given 18 cc of Pyelodil and of a 35 per cent solution of the diethylamine salt by intra venous injection on successive days Blood samples were taken 15 45 and 75 minutes after the injection and urine was collected by catheter for three half-hour periods. X ray films were taken at 3 5 15 30 45 60 and 90 minutes after the injection Iodine was estimated in plasma and urine by Kendall s method The results are given in the table.

a) *Iodine in plasma and urine* In the first patient the plasma iodine and the total amount of iodine in the corresponding urine periods for the two preparations are very similar The second patient showed a lower level of plasma iodine with the diethanolamine salt than with the diethylamine salt and relatively more iodine was excreted in the first half-hour this difference was related to a diuresis produced by drinking tea half an hour before the injection The most notable point in the clearance ratios is that although a high proportion of the iodine was excreted during the first half hour the iodine clearance was less in that period this phenomenon was due to self depression of the iodine clearance at high plasma levels

b) *Radiological appearances* In the first subject it was not possible to differentiate the radiograms obtained by the two preparations. A faint shadow was present as early as three minutes after the injection dye could still be seen in the renal pelvis after 90 minutes In the second patient the films with the diethanolamine salt were much the weaker but this like the lower plasma level and higher clearance may be attributed to diuresis induced by drinking tea before the test It would seem important to practice fluid restriction before pyelography by organic iodine derivatives

TABLE 1

PREPARATION USED	PERIOD	PLASMA IODINE	URINE IODINE	IODINE CLEARANCE
Patient 1				
		mgm per 100 cc.	*mgm per ½ hour*	*cc. per minute*
Diethylamine salt	1	22 8	814	120
	2	6 8	1323	650
	3	2 9	477	540
Diethanolamine salt	1	23 5		
	2	6 4	1089	565
	3	3 0	407	450
Patient 2				
Diethylamine salt	1	14 3	1505	350
	2	4 3	678	530
	3	1 9	333	580
Diethanolamine salt	1	11 4	1618	475
	2	2 6	517	665
	3	1 1	203	615

* The iodine clearance is the number of cc of plasma which would be totally cleared of iodine in a minute, and is obtained from the formula $U/P \times t$, where U is the total amount in mgm of iodine excreted in the urine in a given period, P the iodine content in mgm of 1 cc of plasma, and t the number of minutes in the period (See Homer W Smith The Physiology of the Kidney, New York, 1937)

Conclusion The diethylamine and diethanolamine salts of *3 5* diiodo-*4*-pyridone-*N*-acetic acid do not differ significantly as regards the plasma level, iodine clearance, and pyelographic appearance after a given dose

We are indebted to Messrs Glaxo Laboratories, Ltd for supplies of Pyelodil and the diethylamine salt

A NOTE ON THE PHARMACOLOGY OF DI(β-CYCLOHEXYL-ETHYL) METHYLAMINE HYDROCHLORIDE (CYVERINE HYDROCHLORIDE)

ROBERT A LEHMAN

From the Department of Therapeutics New York University College of Medicine

Received for publication October 27 1940

In a recent publication Blicke, Zeanty and Monroe (1 2) described the preparation of some 60 secondary and tertiary amines and gave a tentative evaluation of their effectiveness as antispasmodics on the basis of the Magnus strip technique The hydrochloride of di(β-cyclohexylethyl) methyl amine, $(C_6H_{11}CH_2CH_2)_2(CH_3)N\ HCl$ was selected from this series as the most desirable compound for therapeutic application It has been put on the market under the trade name cyverine hydrochloride and has been recommended for the treatment of various spastic conditions of the gastro-intestinal tract No reports have been published to date on the action of this drug on the motility of the gastro-intestinal tract of the intact animal and it was therefore thought desirable to put the following observations on record

ACTION OF CYVERINE ON THE MOTILITY OF THE INTESTINAL TRACT

1 An aqueous solution containing 0 01 to 1 0 per cent of cyverine hydrochloride was added to the magnesium free oxygenated Ringer Locke solution in which a 2 cm strip of isolated rabbit or cat jejunum was suspended according to the usual procedure of Magnus. It was found that concentrations of about 1 200 000 for the cat and approximately 1 500 000 for the rabbit jejunum would cause complete paralysis Similar values were obtained by Geiter (3) A further increase in the concentration of cyverine to 1 2 000 stimulated the cat intestine to renewed activity but this observation could not be duplicated with the rabbit jejunum. The effect cannot be ascribed to changes in the pH of the bath since this was determined with the glass electrode and found to vary by not more than 0.3

2 Cyverine was applied topically to the jejunum of intact cats and was found to depress in concentrations of 1 100 000 and to stimulate when the concentration was raised to 1 10 000 These results are in agreement with those obtained on excised tissue

3 Cats and rabbits were anesthetized with chloralosane and dogs with pentobarbital sodium and the method of Babkin (4) as modified by Dreyer

(5) was applied for studying the movements of the jejunum and colon The results of intravenous injection of 0 5 to 6 mgm per kilogram of cyverine hydrochloride are summarized below It should be noted that the relationship between size of dose and effect produced was equivocal in most cases In a number of instances the animals were atropinized but this did not appear to modify the effect of cyverine

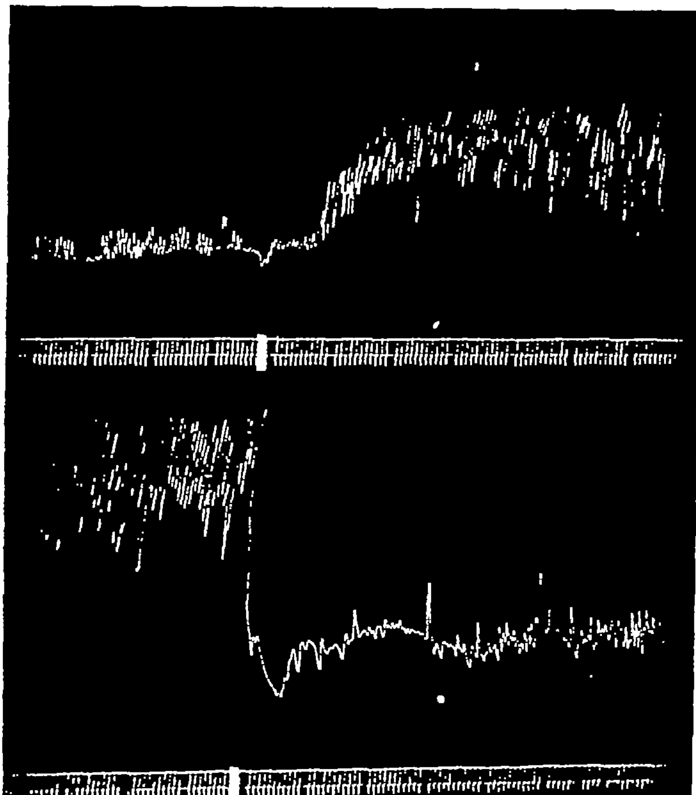

FIG 1 COMPARISON OF THE ACTION OF CYVERINE HYDROCHLORIDE ON THE MOTILITY OF THE JEJUNUM OF TWO DIFFERENT CATS UNDER CHLORALOSANE ANESTHESIA At each signal 4 mgm of cyverine was injected intravenously

Cat For the most part cyverine caused a marked rise in "tone" and increase in the amplitude of the pendular movements of the jejunum In a number of cases the effect was quite the opposite and equal doses of cyverine were found to produce paralysis with a fall in tone (see fig 1) The colon almost invariably showed a rise in the relaxation level and sometimes an increase in the amplitude of the peristaltic waves

Rabbit Marked increase in motility of both jejunum and colon was recorded in every case with usually a rise in tone '

Dog The effects of cyverine on the jejunum and colon of the dog were qualitatively similar to those observed on the cat but were perhaps even less predictable

4 Cyverine hydrochloride was administered in doses of 2 to 5 mgm per kilogram subcutaneously and 2 to 20 mgm per kilogram orally to unanesthetized dogs with Thiry Vella fistulae Changes were observed in the bolus propulsion time according to the method of Quigley but were too inconsistent to allow any conclusions to be drawn Papaverine hydrochloride was administered to test the adequacy of the procedure and was found to cause a marked and uniform slowing of propulsion

OTHER EFFECTS OF CYVERINE

It has been noted that the minimal intravenous dose of cyverine which will affect the motility of the intestinal tract of the cat is 0 5 mgm. per kilogram For comparison the intravenous dosages in milligrams per kilogram which produce other effects are approximately death 15 to 20 convulsions 10 to 15 disturbances in interventricular conduction 5 fleeting fall in blood pressure of 10 to 20 mm of mercury 0 5

SUMMARY AND CONCLUSIONS

It is concluded on the basis of experiments in several species and by various techniques that the marked spasmolytic action of low concentrations of cyverine on excised tissue is complicated by other factors at high concentrations and in the intact animal. In view of the stimulation of the intestine which was frequently observed and in the absence of objective evidence in its favor there seems to be little justification for the clinical use of this drug

The author wishes to express his thanks to Professor A. C DeGraff under whose direction this study was carried out

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CYVERINE HYDROCHLORIDE, A NEW SYNTHETIC PAPAVERINE-LIKE COMPOUND

A REPORT ON ITS VASODILATOR ACTION IN CHRONIC OCCLUSIVE PERIPHERAL VASCULAR DISEASES[1]

ROY J POPKIN

From the Department of Peripheral Vascular Diseases, Outpatient Clinic, Cedars of Lebanon Hospital, Los Angeles, California

Received for publication November 1, 1940

The problem of vasodilators in chronic occlusive peripheral vascular diseases is difficult Rigid, fibrosed, sclerotic vessels as found in thromboangiitis obliterans and arteriosclerosis obliterans do not readily dilate Endothelial thickening, calcium and lipoid deposits or thrombi may occlude the vessel lumen They tend to counteract any increase in caliber Vasospasm is a minor factor except in early cases Inhibition of spasm of the irritated segment and of the reflexly involved collateral and neighboring vessels does offer clinical improvement However, due to the progressive nature of the obliterative disease process in the majority of cases, this element loses its importance

This difficulty explains the lack of unanimity among the workers regarding the choice of a vasodilator drug Literally dozens have been recommended of which many have definite pharmacological activity The final criterion of beneficial action is an increase in blood flow and volume to the affected extremity The site of action may be directly on the higher centers, the vasomotor nerves and ganglia, the smooth muscle of the vessel wall or on the capillaries Important vasodilators recommended in the past few years are ethyl alcohol (1, 2, 3), prostigmine (4), papaverine (5, 6, 7), theobromine compounds (8), calcium (9), tissue extracts (10, 11), sodium chloride (12, 13, 14), histamine (15), and mecholyl (16, 17) Many of these preparations have been found unsatisfactory when used by other workers Lack of consistent results and difficulty of application have limited the usefulness of many Beneficial drugs as mecholyl and histamine require special apparatus and technique Calcium must be given intravenously to obtain an appreciable dilating action The theobromine compounds have not proved consistent Tissue extracts must be given by injection and they have proved disappointing Salt orally has likewise proved of little value Many

[1] The cyverine hydrochloride was kindly supplied by Frederick Stearns and Company

TABLE 1

Experimental observations

Respiratory and pulse rates, oral temperature and blood pressure did not change or changed only slightly they were unchanged or minimal unless otherwise stated

CASE	AGE	SEX	TOTAL DOSE	DIAGNOSIS	OTHER TREATMENTS	REACTIONS	CLINICAL RESULTS
1	[illegible]	M	[illegible] capsules in 4 weeks	Thromboangiitis obliterans	Sitz baths and postural exercises	Very severe persistent heartburn	Intermittent claudication and paresthesias progressively worse during treatment. Oscillometer and surface temperature determinations showed slight but persistent reductions from week to week
2	32	M	120 capsules in 3 weeks	Thromboangiitis obliterans	Sitz baths and postural exercises (Lumbar and cervical sympathectomies 2 years previously)	None	No change. Deeply cyanotic fingers were unaffected
3	31	M	120 capsules in 14 days	Thromboangiitis obliterans	Sitz baths, intermittent venous occlusion, short-wave diathermy postural exercises, mecholyl by ionization	None	No change
4	73	F	45 capsules in 11 days	Arteriosclerosis obliterans	Sitz baths, diathermy and intermittent venous occlusion. Postural exercises	Severe heartburn	No change
5	60	M	120 capsules in 4 weeks	Arteriosclerosis obliterans	Sitz baths and postural exercises	Dizziness and heartburn	Walking distance increased from 2 to 4 blocks. Dull aching pains in calves which previously persisted rest, cleared up. Surface temps. and oscillometer readings unchanged. Surface mottlings over the legs unchanged Patient states he is definitely improved
6	45	M	140 capsules in 3 weeks	Thromboangiitis obliterans	Sitz baths and postural exercises	None	Patient stated he felt better Surface temperatures were slightly increased. Oscillometer readings were decreased
7	71	M	120 capsules in 3 weeks	Arteriosclerosis obliterans	Postural exercises	Dizziness and headache and heartburn	No change. Patient is also hypertensive. Blood pressure 210/120 unaffected

TABLE 1—*Concluded*

CASE	AGE	SEX	TOTAL DOSE	DIAGNOSIS	OTHER TREATMENTS	REACTIONS	CLINICAL RESULTS
8	60	F	160 capsules in 4 weeks	Arteriosclerosis obliterans (diabetic)	Postural exercises	Dizziness and headache and heart burn	No change. Gangrene of a toe developed 2 weeks after cessation of drug
9	62	M	7 capsules	Arteriosclerosis obliterans	Postural exercises	Developed a very severe heartburn and refused further medication	
10	49	M	9 capsules	Thromboangiitis obliterans	Postural exercises	Developed severe dizziness and refused further medication	
11	59	M	26 capsules in 4 days	Arteriosclerosis obliterans	Postural exercises	Developed severe dizzy spells requiring 5 days rest in bed Refused further medication	
12	22	F	This is a normal who was checked before and after smoking, with and without cyverine. Observations recorded at rest and immediately after smoking 2 cigarettes. After a smoking-free period cyverine was given 5 capsules one day and 3 the following morning Observations were checked subject then smoked 2 cigarettes and readings again taken The pulse showed a slight increase on smoking The amplitude of pulsations in the arms and ankles decreased on smoking approximately 1 division point. Readings did not go above normal with cyverine On smoking during the period of cyverine ingestion the amplitude of pulsations decreased but not to as low a level as with smoking without cyverine Surface temperatures decreased on smoking approximately 4 C each time. Cyverine had no effect There were no uncomfortable reactions					

patients cannot take it Intravenously, as the hypertonic solution, its administration is wearing on the patient and its activity is brief Papaverine, of marked benefit in acute cases as embolic occlusion, is of little value in chronic cases Only alcohol and prostigmine remain as vasodilators which can be taken orally over extended periods with desired results

Recently, a synthetic papaverine-like drug, cyverine hydrochloride (18), was introduced It is chemically related to papaverine and pharmacologically its action on smooth muscle is said to be similar In view of the possibility that this new drug could be given orally, it was subjected to clinical investigation

METHOD

Twelve individuals, one normal and eleven suffering from arteriosclerosis obliterans or thromboangiitis obliterans were given the drug Three of these cases were also given cyverine locally by ionization The oral dose varied from three to twelve 20 mgm capsules daily Each cases was followed for approximately four weeks Determinations were made under standard conditions of rest for three-quarters to one hour in a draft-free, constant temperature room Blood pressure, oral temperatures, surface temperatures and oscillometer readings were taken at weekly or semi-weekly intervals Evidences of subjective and objective improvement were noted Readings

were taken from one to three hours after the ingestion of the morning dose of the drug

Patients numbers 2 and 3 were each given 66 grains of sodium nitrite intravenously according to the method of deTakats (19) Each showed an increase in oscillometric amplitude denoting an element of spasm. In Case 2 the pulse in the left ankle increased from 5 8 division points to 8 2 after the sodium nitrite. With cyverine it steadily decreased to 4.2 at this point readings were discontinued Three weeks later the amplitude was back to 5 8 Case 3, showed an increase from 1 0 to 1.8 division points with sodium nitrite. With cyverine no increase was obtained

Cyverine was also applied by ionization to cases 2 3 and 7 during the period of oral ingestion. Forty milligrams were dissolved in 30 cc of distilled water and applied through the positive pole to the affected extremity The applications were given up to thirty minutes at thirty milliamperes. Each patient received five treatments with absolutely negative results

COMMENT

Of the twelve cases, eight suffered from mild to severe persistent heartburn. Dizziness and headaches were less frequently noted. These unpleasant symptoms were so severe that three patients refused further medication Of the eleven cases in which it was tried therapeutically only two stated that there was any subjective improvement. In only one case was there an increase in walking distance.

The lack of results is not surprising Papaverine, which cyverine resembles is of little value orally Its best action is when administered parenterally Investigations of the pharmacological action of papaverine consist mainly of perfusion studies on isolated muscle segments Many cyverine studies have been likewise carried out

It is realized that this series of cases is very small Blood flow and volume determinations were not done However the preponderance of unpleasant symptoms and almost complete lack of objective and subjective improvement halted further trial

SUMMARY

1 Cyverine hydrochloride administered orally frequently causes unpleasant symptoms as heartburn headaches and dizziness

2 Its vasodilator action as shown by surface temperatures, oscillometer readings and clinical improvement is negligible

3 It is not recommended as a vasodilator in chronic obliterative peripheral vascular diseases

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THE PIAL CIRCULATION OF NORMAL, NON ANESTHETIZED ANIMALS

PART 1 DESCRIPTION OF A METHOD OF OBSERVATION[1]

THEODORE P SOHLER, GLADYS N LOTHROP AND HENRY S FORBES

From the Departments of Neuropathology and Neurology Harvard Medical School Boston

Received for publication January 7 1941

In 1938 Clark and Wentsler described a method by which it was possible to make daily observations for weeks or months on the pial circulation of a normal rabbit (1) The advantage of this method was that it enabled an observer to study directly the blood supply of the cerebral cortex after the immediate effects of the operation had subsided and without the complication of anesthesia Nevertheless several difficulties were apparent In the first place with this technique observation was limited to one region of the cortex for the chamber was so large that it had to be placed in the mid-line of a rabbit s skull where the field of view was partly obstructed by the sagittal sinus Secondly the chamber was designed for use with rabbits only Without modification it was not applicable to other animals and the pial arterioles of rabbits were so delicate that operative damage was difficult to avoid Lastly accurate measurements of vessels proved difficult unless the skull was clamped rigidly and this could not be done without anesthesia.

In order to overcome some of these obstacles the following technique has been adopted. Cats and monkeys have been selected as experimental animals and light general anesthesia is used for the primary operation. The fasting animals are given nembutal—0 6 cc of a 6.5 per cent solution per kilogram—by intraperitoneal injection. About thirty minutes before trephining an injection of sterile 30 per cent NaCl (4 cc per kilogram) is made into the peritoneal cavity The purpose of this is to dehydrate the animal so that when the skull is opened the surface of the brain will fall back a little from the inner table of the skull and make it easier to get at the dura without injuring the cortex. The head is now shaved and tincture of metaphen applied to the scalp after a preliminary cleansing with alcohol. Instruments are sterilized by boiling, and sterile gloves gowns towels and gauze masks are used by operator and assistant A sterile cloth is stitched to the scalp over the operative field and a linear incision made through scalp and muscle usually in the parietal region In the cat it is necessary to remove the upper half of the temporal muscle but in monkeys only fascia and periostium are found in this region The operative field is again swabbed with metaphen, including the exposed surface of the skull

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[1] This study was aided by a grant from the Institute for the Study of Analgesic and Sedative Drugs.

The skull is now trephined and any bone bleeding stopped with sterile bone wax A stainless steel tap, slightly bevelled, is screwed into the hole in the skull and then removed, to make a track in the bone All loose spicules of bone are removed by forceps from the inner table of the skull, and small clots (or bloody fluid) are wiped gently from the cut surface of the bone by means of a small pledget of cotton moistened with Ringer's solution The next step is to pick up the dura with the point of a needle, incise it and remove a circle of dura by a wire cautery, carefully lifting the membrane away from the brain with fine forceps The cautery is of fine nichrome wire heated to a dull red, and care should be taken not to hold it near the cortex for more than a few seconds at a time No instrument or cotton is allowed to touch the exposed arachnoid, and speed is necessary to avoid drying or irritation by cool air currents Warm sterile Ringer's fluid is gently flooded over the surface of the brain the moment the circle of dura is removed As soon as possible a circular window of lucite, moistened on its under surface with Ringer's, is screwed into the hole in the skull

The cranial window now in use requires special description as it differs from the one which we have used in acute experiments A circular disc of "lucite"[2] (methyl metha-

FIG 1 CRANIAL WINDOW

Adapter for injecting Ringer's and expelling air bubbles is screwed into upper hole Machine screw by which window is sealed is in lower hole

crylate), 4 mm thick for a monkey and 6 or 7 mm for a cat, is machined with a fine thread, slightly bevelled, so as to fit into the hole trephined in the skull The diameter of the disc should be such as to fit exactly the hole made by the individual trephine drill A disc of 16 mm diameter has proved to be the most serviceable size, though one of 12 mm gives a wider choice of positions on the skull where it can be placed The under surface of the disc is made slightly concave to conform to the curvature of the skull, the upper surface is flat and both are highly polished so as to make of the disc a trans parent window Two small depressions in its top, near the rim, fit a special wrench for screwing the window into the skull, and two small holes are made through the disc, also near the rim These holes, to inject Ringer's solution and expel air bubbles beneath the window, are threaded so that they may be sealed by small machine screws

After the window is in place, the air beneath it is replaced by warm Ringer's solution, the holes sealed, and the junction between rim of window and skull is checked for pos

[2] The lucite used for these windows is of a special composition which can be sterilized by boiling without turning opaque Often it is desirable to polish the surface of the window with Simoniz just before observations are to be made

Fig 2. Cat—under Anesthesia—at End of Operation Window Installed and Ready for Gauze Dressing

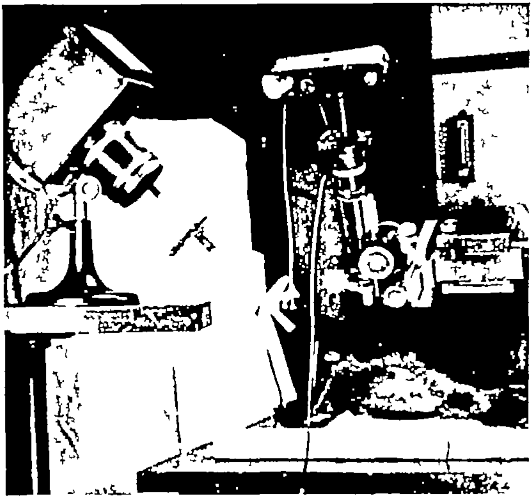


Fig 3 Monkey Ready for Observation—No Anesthesia—Lying on Table with Arms and Legs Tied Microscope with Mechanical Stage Camera above and Light Source on Left

sible leaks If a leak is found the window may be unscrewed and reinserted at a slightly different angle

Where the edges of the incision overlap the window a half circle of scalp is removed on either side so that the scalp will fit closely about the window Where the incision extends beyond the window the edges of the scalp are approximated and held by loose interrupted sutures, thus no purse string sutures are needed to hold the scalp in contact with the rim of the window Metaphen is applied to the wound surface The final dressing consists of powdered thymol iodide, a sterile gauze pad and a sleeve of elastic stockinette drawn over the head and two holes cut for the cat's ears For a monkey a soft leather helmet is substituted for the stockinette

The animals begin to wake up soon after the operation but usually they are sleepy for 12 to 24 hours and it is important—especially with monkeys—to keep them warm during this period

For observation a microscope mounted on a holder with a two-way mechanical adjustment is swung out over the animal's head from an extra heavy desk clamp A beam of light from a 165 watt filament lamp is filtered through a green solution (copper sulphate plus picric acid) and is focussed on the cranial window The animal is trained by petting and feeding to lie quietly on the table, and its head is held gently by hand It is often helpful to blindfold cats with a band of cloth To get objective evidence of change (or lack of change) in the diameter of blood vessels photomicrographs taken at frequent intervals have proved useful The usual exposure has been one tenth of a second, with 30x magnification taken on 35 mm film The enlarged prints can then be studied at leisure [3]

OBSERVATION ON THE GROWTH OF NEW TISSUE FROM THE CUT EDGES OF THE DURA, UTILIZING THE PRESENT METHOD OF STUDY

Sometimes as early as five days after operation (though usually much later) a thin film of tissue may be seen growing over the arachnoid from the periphery This tissue—supplied with slender vessels arising from the dura—grows toward the center of the window rather symetrically from all points, and gradually increases in thickness until the underlying pial vessels are entirely hidden (see fig 4) Occasionally, however, vessels from both pia and dura may form a network growing in irregularly from scattered points, and the opaque film of tissue between the vessels increases until the pial vessels below are obscured Then again, a few vessels may develop in small isolated areas—probably at the sites of pial injury Here they continue to grow locally without spreading further

In one cat the newly formed membrane covering the arachnoid was removed by electro cautery after the window had been in place for three months No adhesions were found between this membrane and the arachnoid, and the membrane itself resembled the dura, but was thicker The pial blood vessels, exposed for a second time, showed almost the same pattern as at the time of the first operation ninety-five days before The window was now reinserted

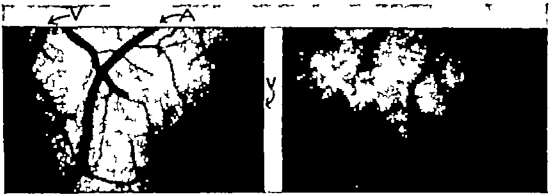
[3] Caution should be used in estimating vessel diameter from prints We have found that if two prints, one light and one dark are made from the same negative blood vessels in the dark print will look wider than the same vessels in the light one

and the field remained clear Some of the vessels, especially the veins, showed fusiform bulgings about ten days later, probably due to slight trauma at the time of this second operation

In a second animal a similar attempt was made to separate the new membrane from the arachnoid, but in this case the two membranes were found to be closely adherent

In several monkeys a second window was installed in the opposite parietal bone after the first one had been forced out of place by an accidental blow No infection resulted and the new window healed in normally The reactions of the pial arteries were the same as before in spite of the bone defect at the site of the old window

As Clark and Wentsler found in rabbits so we have noticed in cats and monkeys that the pial vessels of all sizes show a remarkable constancy of



A B

FIG 4. A monkey normal appearance of cortex (8 days after insertion of window) B monkey, 32 days after insertion of window—new vessels are growing in from edges of dura. Different field from that shown above

calibre and of blood flow under normal conditions, even during periods of emotional excitement

Up to the present time the operation has been carried out successfully in twenty four animals—eighteen cats and six monkeys and the window has stayed in place for periods of from one week to four and a half months.

One difficulty has not yet been fully overcome and that is the measuring of small changes in diameter of the vessels. To accomplish this a magnification of 100× is desirable and the head should be immobilized in a clamp but that in turn requires a general anesthetic Furthermore without greatly disturbing the animal one cannot get continuous records of blood pressure, cerebrospinal fluid pressure etc On this account the usefulness of the method seems limited at present to the recording by low power photographs of big changes in vessel calibre possibly also to the study after experimental embolism of the manner in which the circulation is re-established and similar problems

SUMMARY

A method is described for prolonged observation of blood vessels supplying the cerebral cortex of non-anesthetized animals Cats and monkeys have been studied so far, but the technique may be applied to other animals

A brief description is given of the outgrowth of repair tissue from the meninges

Some of the limitations of the method are mentioned

We wish to thank Miss Joan Wilkinson for technical assistance

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THE PIAL CIRCULATION OF NORMAL, NON ANESTHETIZED ANIMALS[1]

PART II THE EFFECTS OF DRUGS, ALCOHOL AND CO_2

THEODORE P SOHLER, GLADYS N LOTHROP AND HENRY S FORBES

From the Departments of Neuropathology and Neurology Harvard Medical School, Boston

Received for publication January 7 1941

The present experiments were undertaken to study the effect of certain drugs on the cerebral circulation of animals without the complication of a previous anesthetic We were especially interested to learn what changes in caliber of the pial arteries took place in response to a single drug The method of investigation was described in Part 1 In order to avoid pain or struggling it was found best to restrain the animal lightly but not to immobilize the head in a clamp or to record blood pressure

RESULTS

Five cats and five monkeys were used in these experiments. The results are summarized in table 1

Avertin caused vasodilation in seven out of ten experiments The maximum dilation was seen about fourteen minutes after the injection Forty minutes or so later the arteries had returned to their former size and the reflexes were beginning to reappear

At the end of an avertin experiment benzedrine sulphate (by intraperitoneal injection) shortened the time of recovery considerably Twice the benzedrine was followed by constriction i e the pial arteries became narrower than they were originally Benzedrine alone—not following avertin—was given in two additional experiments. No vascular change was seen

Neither dial nor nembutal caused any significant changes of vessel caliber in these experiments[2] In previous experiments on anesthetized animals successive doses of dial had been followed by slight dilation (1) The conditions in the two groups of experiments, however were not identical for the anesthetized group were observed immediately after the operation of

[1] This study was aided by a grant from the Institute for the Study of Analgesic and Sedative Drugs

[2] Two of these animals with 'permanent windows were given anesthetic doses of dial and of nembutal respectively before the test injection In these cases (in the presence of anesthesia) no dilation took place

installing the cranial window, while the "non-anesthetized" group had fully recovered from this operation

Ethyl alcohol (25 per cent solution) seemed to have a very different effect on these animals with "permanent" windows from what it had in acute experiments on animals under dial anesthesia In the latter animals alcohol, given intravenously or by stomach, had caused prompt vasodilation (2)

TABLE 1

All of the animals had "permanent" cranial windows

DRUG	DOSE	SITE OF INJECTION	NUMBER OF EXPERIMENTS	NUMBER OF EXPERIMENTS SHOWING THE FOLLOWING EFFECTS ON PIAL ARTS		
				Dil.	Const.	No change
"Avertin fluid"*	4–6 cc per kgm	Rectum	2	2		
Solution of avertin crystals†	4–13 cc per kgm	Rectum	4	1		3
Solution of avertin crystals†	6–8 cc per kgm	Perit	4	4		
Avertin totals			10	7		3
Dial‡	35– 55 cc per kgm	Perit	1			1
Dial‡	04– 07 cc per kgm	Vein	4			4
Nembutal§	4– 7 cc per kgm	Perit	4		1	3
Alcohol (25 per cent)	12–28 cc per kgm	Stom	4			4
Alcohol (25 per cent)	2–6 cc per kgm	Vein	3		1	2
CO_2	10 per cent	Lungs	8	8		
Benzedrine (after avertin)	2–7 mgm per kgm	Perit	7		2	5
Metrazol	8–15 mgm per kgm	Vein	4	2		2
Acetanilide	1700–2000 mgm per kgm	Stom	2			2

* Avertin fluid—tribromethanol (sat sol) in amylene hydrate diluted with water to make a 2 5 per cent sol of the original "fluid " 1 cc contains 25 mgm avertin

† Avertin crystals—2 5 per cent solution in distilled water

‡ Dial (contributed for experimental purposes by the Ciba Co , Lafayette Park, Summit, N J) each cc contains diallylmalonylurea, 0 1 gram urethane, 0 4 gram and monethylurea in distilled water

§ Nembutal—pentobarbital sodium (alcohol 10 per cent)—each cc contains 65 mgm nembutal

In seven experiments on animals with "permanent" windows there was no immediate change in vessel caliber Twenty hours later, it is true, two of the monkeys, which had been given alcohol by stomach tube and had become stuporous, showed congestion of the cortex But this delayed reaction was different from anything seen previously

Carbon dioxide, a strong vasodilator in acute experiments on anesthetized

FIG 1 SOLUTION OF AVERTIN CRYSTALS

1	2	3	4
Control	30 sec. after injection	11 min. after injection (asleep)	22 min. after injection (waking)

Photomicrographs of pial vessels before and after an intraperitoneal injection of a 2.5 per cent solution of avertin crystals (8 cc. per kilogram) The artery (*a*) is dilated in 2 and more dilated in 3 In 4 it is nearly back to its original size The window (the second which had been installed in this monkey) had been in place twenty-one days at this time Some new vessels which were growing in over the arachnoid are seen on the left side of the field.

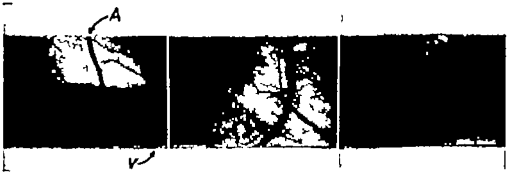

FIG 2 AVERTIN FLUID

1	2	3
Control	4 min. after injection (asleep)	25 min. after injection (asleep)

Photomicrographs before and after a rectal administration of avertin fluid (6 cc per kilogram) The pial artery (*a*) is dilated in 2 and is back to its original size in 3 The window has been in place in this monkey for fourteen days.

There was found to be no constant relationship between the return of the artery to its normal diameter and the recovery of consciousness. Compare figures 1 and 2.

FIG 3 DIAL

1	2	3
Control	17 min. after injection (asleep)	46 min after injection (still asleep)

Photomicrographs before and after an intraperitoneal injection of dial (0 4 cc. per kilogram) The cortex was observed and photographed constantly for seventy minutes following the injection There was no change in the cerebral vessels during this period. The window had been in place in this monkey for eighteen days and new vessels were beginning to grow in over the arachnoid

animals (3), caused vasodilation also in these experiments In five additional experiments (not recorded in table 1) carbon dioxide was given both before and after anesthesia The result in every instance was dilation

Metrazol had caused strong vasodilation in acute experiments on anesthetized animals (4) In the present series photographs were taken at frequent intervals, beginning five seconds after the injection and continuing for six minutes No significant dilation occurred [3]

Experiments on anesthetized animals had shown that acetanilide, if given in small amounts, caused no consistent change in pial vessel caliber A conspicuous change which did occur was a peculiar brownish cyanosis of the pial circulation The color change was noticed about eighty minutes after a small intravenous dose (as small as 10 mgm per kilogram) (1) The results were similar in the present experiments In one monkey with a "permanent" window acetanilide (500 to 1400 mgm) in 2 per cent gum arabic solution was given daily by stomach tube for six days The total amount of

FIG 4 ALCOHOL

1	2	3	4
Control	During injection	1 5 min. after injection	7 min after injection

Photomicrographs before and after an intravenous injection of alcohol (25 per cent—3 5 cc per kilogram) There was no change in diameter or appearance of the pial vessels either during or after the injection (watched for 34 minutes) The window (the third which had been installed in this monkey) had been in place thirteen days at this time

acetanilide given during this time was 1700 mgm (per kilogram) Another monkey was given 2000 mgm per kilogram in similar manner during a period of four days One of these animals showed some constriction of the pial arteries during the first three days, after which the vessels returned to their former size The other animal (with many new vessels partly obscuring the pial arteries) showed no definite change in vessel caliber In both animals cyanosis appeared on the second day and became more evident on the fourth and fifth

COMMENT

Several drugs appeared to have a stronger vasodilator action on animals under general anesthesia than on those with no anesthesia This difference

[3] Among these animals with 'permanent' windows was one which received an anesthetic dose of dial before the metrazol In this case as with the conscious animals there was no significant vasodilation

in all probability was due not to the anesthesia itself but to another factor—the recent exposure of the pial vessels of the "anesthetized" group to operative trauma. In the "non-anesthetized" group the vessels had long since recovered from such exposure and were protected against sudden changes. The significant feature indeed of this latter group was not the absence of anesthesia but the presence of a permanent cranial window.

The difference in results between the two groups of animals might be given a still different explanation: it is possible that the recently exposed vessels were not more reactive but that the protected vessels—long in contact with a lucite window—had become less reactive than normal. There seems, however, to be no good evidence for this supposition. The vessels beneath the "permanent" window showed no signs of injury and they reacted normally in other ways—by dilation in response to CO_2 and by a weak constriction after stimulation of the cervical sympathetic nerve.

Without data on blood pressure to supplement the observations on the cerebral arteries it is impossible to estimate even qualitative changes in cerebral blood flow. It should be stressed that dilation of these arteries is not in itself proof of increased blood flow through the brain, nor is constriction proof of decreased flow. Changes in rate of flow depend chiefly upon changes in blood pressure (5).

The rate of blood flow (i.e., the observed movement of corpuscles through the small venules of the pia) was not noticeably altered in these conscious animals by emotional disturbances such as anger, fear or excitement. Great excitement with muscular movements, however, made observation by microscope impossible, and the statement just made refers to milder states.

SUMMARY

In animals with permanently installed cranial windows the effects of certain drugs on the pial arteries were as follows:

Avertin and carbon dioxide caused vasodilation.

Alcohol, dial, nembutal, metrazol and acetanilide were followed by little if any immediate change in vessel caliber.

Acetanilide, by mouth, caused cyanosis visible in the pial circulation.

The presence or absence of general anesthesia did not appear to influence the results noticeably, but recent operative exposure seemed to increase the dilator effect of some drugs.

We wish to thank Miss Joan Wilkinson for technical assistance.

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THE FIBRILLATION THRESHOLD AFTER ADMINISTRATION OF DIGITALIS AND OUABAIN[1]

RENÉ WÉGRIA,[2] J H GEYER AND B S BROWN

From the Department of Physiology, Western Reserve University Medical School, Cleveland, O

Received for publication February 11, 1941

A single toxic dose of digitalis or ouabain, or successive administrations of smaller doses to dogs, cause death, usually as a result of ventricular fibrillation The fibrillation is preceded by premature ventricular beats which arise from various foci and may occur isolated or grouped, often eventuating in runs of ventricular tachycardia (1–4) The mechanism by which such fibrillation is initiated is of importance not only in its relation to fibrillation induced in other ways, but also because the onset of ventricular fibrillation represents the ultimate standard upon which the bio-assay of digitalis really depends in mammals and, unless such fibrillation is dependent upon a specific physiological mechanism, it cannot be very exact The possibility therefore deserved exploration whether this may prove to be a fundamental reason why dogs are not generally regarded as favorable animals in the bio-assays of digitalis (3)

The arrhythmia which precedes ventricular fibrillation after digitalis resembles so closely that which frequently precedes ventricular fibrillation due to coronary occlusion that a similar mechanism may be suspected In regard to the latter, Wiggers, Wégria and Piñera (5) recently found that, in experimental coronary occlusion, the vulnerability of the dog ventricles to a short direct current stimulus applied during late systole, is increased, i e , ventricular fibrillation can be induced by a weaker direct current shock in a heart with an ischemic area than in a normal one Since ischemia reduces the fibrillation threshold to artificial stimuli and also causes the appearance of ectopic foci, they put forward the theory that spontaneous ventricular fibrillation during coronary occlusion may be due to those ectopic stimuli, now of threshold value for the hypersensitive myocardium, anyone of them being able to induce fibrillation when it falls during the vulnerable period of either a normal beat or that of an extrasystole

[1] This investigation was supported by a grant from the John and Mary R Markle Foundation

[2] Fellow of the Belgian American Educational Foundation

The present research was started to determine whether digitalis and ouabain have a similar effect on the fibrillation threshold The individual physiological basis upon which determination of such threshold depends, has been presented in previous papers (6–8) by Wiggers and Wégria and may be briefly reviewed with the aid of the diagram of figure 1 If a short rectilinear shock (d) or (e) of moderate strength and 0.02 second in duration is applied to the ventricle during diastole a premature ventricular systole is produced If an identical shock (a) is applied during the pseudo-refractory period of late diastole it is ineffective so is any identical shock (b) applied during the absolute refractory period of ventricular systole labeled R But if an identical shock (c) is

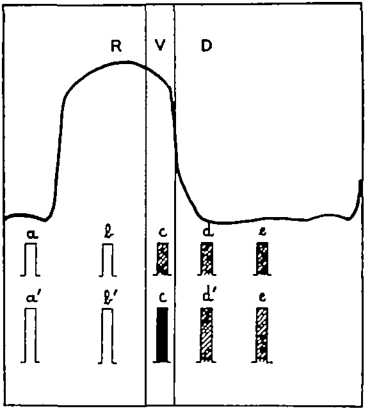

FIG 1 The upper tracing, a left intraventricular pressure curve R, absolute refractory period V vulnerable period D diastole a, b c, d e short direct current stimuli all 0 02 second in duration a b[1] c[1] d[1] e similar but stronger direct current stimuli

applied during the so-called vulnerable period V extending over late systole and probably the first moments of protodiastole it may produce one—sometimes two—premature ventricular systoles

If shocks a[1] b[1] c d[1] e[1] stronger and 0 02 second in duration are applied respectively in the same periods of the heart cycle as shocks a, b c d e shocks d[1] and e[1] however strong they may be will produce one ventricular extra-systole and never two premature systoles or ventricular fibrillation Shocks a[1] and b[1] however strong they be will be ineffective But shock c[1] may produce ventricular fibrillation The strength of such a short rectilinear shock, 0 01 to 0 03 second in duration just sufficient to cause the ventricle to fibrillate when applied to the ventricular surface at the right moment of the heart cycle, has been termed the "fibrillation threshold (9) To study the action of physiological pharmacological or pathological factors on the fibrillation threshold

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noticed numerous ventricular extrasystoles but afterwards the heart rate became regular again and the beat of sinus origin. The S-T segment, how

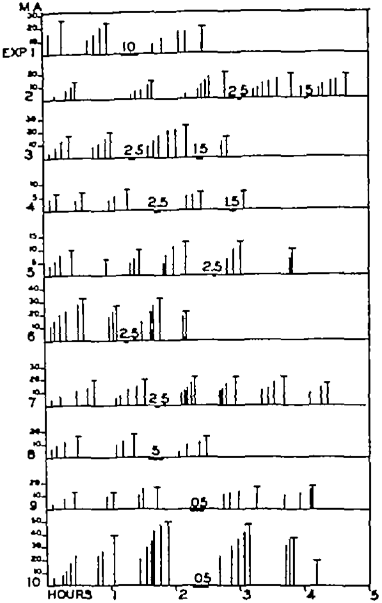

FIG 2. PLOT SHOWING THE FIBRILLATION THRESHOLD OF THE LEFT VENTRICLE BEFORE AND AFTER DIGITALIS AND OUABAIN

Each vertical line represents the strength in milliamperes of each shock tested a shock which produced fibrillation is indicated by the sign *T* In experiments 1 to 8 included, the numerals refer to the number of cc. of digitalis infusion injected per kilogram of animal body weight. In experiments 9 and 10 the numerals refer to mgm. of ouabain injected per kilogram of animal body weight. The heavy line under those numerals represents the duration of the injection Duration of shock experiments 1 and 6, 0 025 second experiments 2, 4 5 9 10 0 020 second experiment 3, 0 026 second experiment 7 0 030 second experiment 8 0 015 second

ever, became more and more depressed the P R interval increased (0 15 second) and the QRS complex was wider In the presence of such clear signs

of digitalis action a fibrillation threshold determination was begun and the same D C stimulus, 0 025 second in duration, was used It had to be progressively increased up to 21 M A to be able to produce ventricular fibrillation at 4 35 p m This experiment shows conclusively that a dose of digitalis sufficient to produce evident signs of digitalis action does not significantly alter the fibrillation threshold

All experiments plotted in figure 2 are experiments in which we had similar clear signs of digitalis or ouabain action It will be noticed that in most of these we obtained no signs of action of the drugs with smaller doses, in which case we very often administered a second dose of digitalis Attempts to study stronger doses of digitalis and ouabain proved unsuccessful due to the development of irregular ectopic rhythms which made our method inapplicable, since the irregularity of the heart under these circumstances prevented certain application of our stimulus at the proper moment of the heart cycle On the other hand, since the fibrillation threshold for an ectopic ventricular beat is perhaps not the same as that of a beat of sinus origin, no conclusions could properly be drawn from such experiments

As shown in plots of experiments 9 and 10 of figure 2, the results obtained with digitalis and ouabain are entirely comparable It should be said for both series of experiments, however, that there may be a progressive slight decrease in the fibrillation threshold after digitalis (fig 2, Exp 6) or ouabain (fig 2, Exp 10) We hesitate to attribute significance to such small changes, particularly as a similar, slight decrease in the threshold occurred in some prolonged experiments in which no drug had been administered It may be added that signs of drug action were not more evident when the fibrillation threshold started to decrease than when the threshold was the same as the control threshold We therefore conclude that digitalis and ouabain (*g*-strophanthin), in doses sufficient to produce the signs of cardiac action we described, do not alter significantly the fibrillation threshold of the dog ventricles

Spontaneous ventricular fibrillation after digitalis or ouabain administration Although the fibrillation threshold of the dog ventricles is not changed by therapeutic doses of digitalis or ouabain, the type of fibrillation produced by a strong stimulus applied during late systole in a digitalized heart is quite different from the fibrillation produced in a normal heart

In a normal heart fibrillation starts with a short undulatory stage followed within 1 or 2 seconds by a period during which the electrocardiographic waves get shorter and shorter and then the heart passes into fine fibrillation In the fibrillation produced by an electrical stimulus in a digitalized heart the waves have a more regular appearance even after one second of fibrillation, they are more uniform in shape as well as in voltage, their frequency is less and they show no tendency to shorten

In a few experiments we gave, in fractionated doses, an amount of digitalis

or ouabain sufficient to produce spontaneous ventricular fibrillation Such ventricular fibrillation is still different The successive changes as shown by figure 3 can roughly be grouped into the following stages

1 Lengthening of P R interval various degrees of A V block depression of S-T segment or inversion of T wave and abnormalities of QRS (fig 3 2–3)

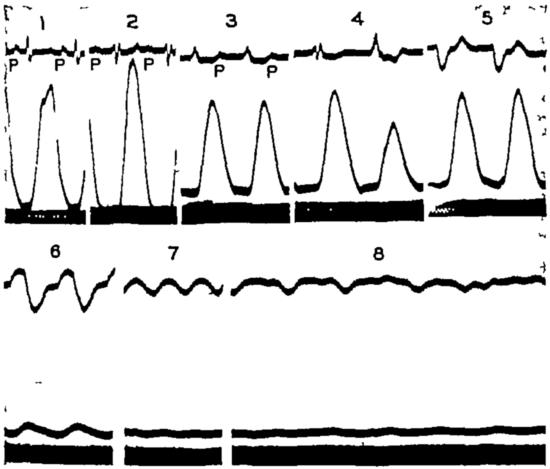

Fig 3 Segments of Records Showing Progressive Changes in E.C.G (III) (Upper) and Left Ventricular Pressures (Lower) following Toxic Doses of Digitalis

1 early effects, essentially normal curves *2* prolongation of the P R interval *3* prolongation P R interval depression S-T segment, deformation of QRS complex; *4* multifocal ventricular rhythm *5* ventricular rhythm *6* ventricular rhythm with intraventricular conduction defect resulting in ineffective beats (see pressure curve) *7* regular ineffective tachysystoles (333/min) *8* start of coarse fibrillation Time $\frac{1}{25}$ sec

2 Development of nodal and ventricular premature beats and paroxysms of ventricular tachycardia with definite elevations of ventricular pressure (fig 3 4 5)

3 Pronounced widening and deformation of electrocardiographic deflections i e low frequency or occasionally temporarily increased frequency—all with very little elevation of intraventricular pressure (fig 3 6–7)

4 Wide irregular E C G complexes without any appreciable ventricular pressure elevations eventuating in true fibrillation (fig 3, 8) The type of fibrillation thus developed resembles more that following toxic doses of KCl (11) than that due to electrical currents (7–8), or coronary occlusion (5)

DISCUSSION

In trying to formulate a conception as to how ventricular fibrillation is produced by toxic doses of digitalis or ouabain, we have considered the possibility that large doses may lower the fibrillation threshold of the ventricles, whereas the therapeutic or subtoxic doses which we studied do not alter that threshold While this cannot be definitely ruled out, we have to recall that in the case of fibrillation produced by coronary occlusion a reduction in fibrillation threshold occurs long before such ventricles go into fibrillation It seems therefore that the explanation of the fibrillation produced by digitalis or ouabain in toxic doses is not to be found in a lowering of the fibrillation threshold by those toxic doses

With the observations of Wiggers and Stimson (12) that digitalis prolongs intraventricular conduction, even in subtoxic doses, it seems more probable that the development of this significantly different type of ventricular fibrillation is the result of changes in myocardial conductivity and possibly in the refractory period In this event, the inception of the stage which can truly be characterized as "fibrillation" must be referred to a favorable development of localized blocks rather than to the advent of an effective stimulus

SUMMARY

1 As a result of previous investigations, a critical method for determining the sensitivity of the dog ventricles to fibrillation is presented It consists fundamentally in measuring the strength of a short D C stimulus applied in late systole, just sufficient to induce fibrillation

2 Studied by this method, digitalis and ouabain (*g*-strophanthin) in doses sufficient to elicit clear signs of action, do not alter significantly such a fibrillation threshold of the dog ventricles

3 Since toxic doses of digitalis and ouabain induce spontaneous fibrillation of the ventricles, some other mechanism must be involved Results are presented which lead to the conclusions (a) that the type of fibrillation induced differs significantly from that caused by electrical currents and coronary occlusion in normal hearts and (b) that its onset and development depend on favorable development of localized blocks and changes in myocardial conductivity and does not require the advent of an effective stimulus during the vulnerable period, as in electrocution and coronary occlusion

4 The results are of fundamental importance in showing (a) that ventricular fibrillation may result as a consequence of more than one mechanism, and (b) that the slow and somewhat variable progression of the fibrillating

process which leads to death from digitalis, is one of the factors which makes dogs unfavorable animals for the bio-assay of digitalis.

We wish to express our sincere thanks to Professor Carl J Wiggers for his continuous guidance throughout the work and to Dr G K. Moe, Messrs. H D Kohn and P M. Kohn for their help in some experiments

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THE REDUCTION OF NEOPRONTOSIL BY TISSUES IN VITRO

FREDERICK BERNHEIM

From The Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina

Received for publication January 30, 1941

It is generally agreed that prontosil and related compounds are reduced in the animal body at the azo linkage and that one of the reduction products is sulfanilamide (1) However, the mechanism by which this reduction is brought about is not known Long and Bliss (2) obtained some reduction by injecting formaldehyde sulfoxalate into rabbits along with the drug Bliss and Long (3) showed that cysteine incubated for twenty four hours with prontosil caused its reduction It seemed probable that the cells contained reducing systems which might be capable of bringing about this reduction more rapidly It was of interest, therefore, to study the reduction of neoprontosil *in vitro* by certain tissues and the effect of the addition of various substrates

EXPERIMENTAL

The tissues of the white rat were used for most of the experiments although similar results were obtained with guinea pig tissue They were removed immediately after killing, chopped with scissors, ground in a mortar with a little sand and M/20 phosphate buffer (pH 6 7) and squeezed through muslin One cc of the resulting suspension was added to 1 0 cc of buffer (pH 6 7) and 0 1 cc of neoprontosil, containing 0 25 mgm , and incubated in a test tube at 37° If the suspension is thoroughly aerated no reduction of the dye occurs If the tissue is incubated anaerobically the reduction is somewhat more rapid than when incubated semi-anaerobically in a test tube, but because of convenience the latter method was used and gave satisfactory results At the end of the incubation period 6 0 cc of 95 per cent alcohol were added to each tube and the tubes centrifuged Addition of the dye to controls just before the addition of the alcohol showed that it was possible to recover all of it by this procedure After centrifugation the clear liquid containing the unreduced dye was decanted and estimated in a colorimeter against a suitable standard dissolved in the same concentration of alcohol

Table 1 shows the amount of dye reduced by liver, kidney and brain in a certain length of time, the wet weight of the tissues in the three cases being the same The liver causes the most marked reduction, the kidney shows a

slight reduction and brain none at all The following experiments were therefore done with liver in order to determine what components in the tissue were responsible for the reduction The liver suspension was diluted to 50 cc and washed by centrifuging This was done two or three times and the resulting solid finally suspended in buffer This material had very little oxygen uptake and when added to the dye under the standard conditions caused very little reduction (table 1) even though the proteins contained free sulfhydryl groups In the next experiment the liver suspension was boiled for 5 minutes before adding the dye This also inhibited the reduction almost completely (table 1) Finally KCN in a concentration of m/200 was added to the suspension The fact that this also inhibited the reduction of the dye completely (table 1) indicated that the oxidation of some substrate

TABLE 1

The reduction of 0.25 mgm of neoprontosil by tissues with various substrates at [illegible]

Unless otherwise indicated the pH was 6 7 The figures represent the [illegible] unreduced at the end of a given time. There was no appreciable reduction of the dye by cysteine or thioglycollic acid alone during the time of the experiment

TISSUE	ALONE	2.0 MGM. CYSTEINE HCl	2.0 MGM. THIOGLYCOLLIC ACID	2.5 MGM. ETHYL ALCOHOL	TIME
	mgm.	mgm.	mgm.	mgm.	hours
Liver	0 14	0 09	0 11	0 12	[illegible]
Liver (pH 7.8)	0 14	0 04	0 11	0.0[illegible]	[illegible]
Liver (washed twice)	0 24	0 06	0 14	0.23	[illegible]
Liver (m/200 NaCN)	0 24	0 23	0 24	0 2[illegible]	[illegible]
Liver (boiled)	0 25	0 24	0 25	0.2[illegible]	[illegible]
Kidney	0 22	0 11	0 14		[illegible]
Brain	0 25	0 24	0 25		[illegible]

by a cyanide-sensitive system was coupled with the reduction of [illegible] tosil.

Various substrates were added to both the washed and [illegible] suspensions to see whether any of them increased the reduction [illegible] included succinate choline *d* amino acids, glucose lactate, [illegible] glutathione cysteine thioglycollic acid and ethyl alcohol [illegible] cysteine thioglycollic acid and ethyl alcohol increased the [illegible] The others had no effect The addition of cysteine and [illegible] kidney suspension increased the reduction rate somewhat [illegible] brain these substances had no effect (table 1) The same [illegible] alcohol caused no reduction when they were added to [illegible] treated with cyanide (table 1) When added to the [illegible] any tissue no appreciable reduction occurred during the [illegible] ment. Either the presence of a thermolabile cyanide-[illegible]

liver specific for the oxidation of SH compounds such as cysteine and thioglycollic acid (but not glutathione), or the catalyst responsible for the oxidation of ethyl alcohol was necessary before these compounds could reduce the dye at a rapid rate The substitution of copper and iron salts for the tissue failed to increase the reduction rate in the presence of cysteine and thioglycollic acid although copper and iron rapidly oxidize these compounds

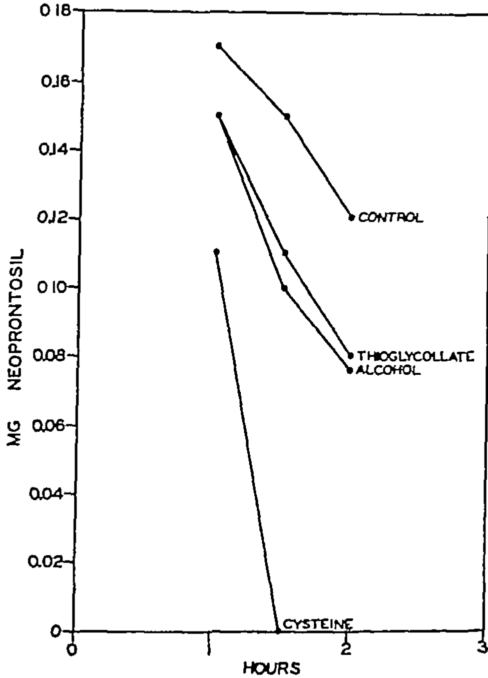

FIG 1 THE REDUCTION OF 0 25 MGM OF NEOPRONTOSIL BY LIVER SUSPENSION WITH AND WITHOUT 2 0 MGM OF THE INDICATED SUBSTRATES

pH 6 7, 37° The figures represent the amount of neoprontosil unreduced at the indicated times

The relative rates of reduction are shown in fig 1 Cysteine causes the most rapid reduction Thioglycollic acid and alcohol reduce at about the same rate All three compounds show a significant increase over the control A similar curve could be plotted for washed liver suspension, although in this case the alcohol is ineffective because the catalyst responsible for its oxidation has been washed away and the time required for complete reduction by cysteine and thioglycollic acid is much longer A change of pH from 6 7 to 7 8 has no effect on the reduction except in the case of alcohol which at the

higher pH reduces more rapidly Thus at the end of an hour 0 14 mgm. of the original 0 25 mgm of neoprontosil was still unreduced in the controls. In the presence of 2 0 mgm. ethyl alcohol, 0 12 mgm were still unreduced at pH 6 7 but at pH 7 8 only 0 04 mgm remained. This might be expected because the activity of the alcohol oxidase is greatest at pH 8 0 whereas the oxidase for cysteine and thioglycollic acid has no sharp optimum in this range. This pH effect is further evidence that the reduction of the dye is dependent on the activity of certain intracellular catalysts.

It should be mentioned that when cysteine is added to liver under these experimental conditions the hemoglobin is converted into a green pigment This does not interfere with the determination as it is precipitated by the alcohol It is also not essential to the reduction as the neoprontosil is reduced by the washed tissue and cysteine when no appreciable amount of hemoglobin is present.

DISCUSSION

As muscle was also inactive the evidence obtained in these experiments indicates that the reduction of the azo dye neoprontosil occurs almost exclusively in the liver and in that organ only certain enzyme systems are involved The somewhat different results of Engel (4) are difficult to explain because he does not give the conditions of his experiments. The question remains whether this specificity manifests itself only with neoprontosil or whether other azo dyes are reduced by the same mechanisms This is difficult to answer because of the extreme insolubility of many of the azo dyes Thus scarlet red was not appreciably reduced by liver but it is doubtful whether much was in solution. Pyridium is more soluble and is reduced but cysteine and alcohol have comparatively little effect on the rate. Chrysoidine, another azo dye, was reduced when fed to animals (5)

SUMMARY

1 A suspension of rat liver incubated semi anaerobically with neoprontosil reduced the dye. Kidney under the same conditions causes comparatively little reduction and brain and muscle none at all.

2. The addition of cystine thioglycollic acid or ethyl alcohol increases the reduction rate of liver but has little effect when added to kidney or brain. Glucose lactic acid amino acids amines, succinic acid, choline aldehydes and glutathione have no effect on the rate of reduction by liver

3 Cysteine and thioglycollic acid alone or in the presence of copper or iron salts reduce neoprontosil only very slowly

4 Boiling or the addition of KCN prevents the reduction by the liver alone and with the substrates. Washed liver reduces very slowly and the addition of cysteine and thioglycollic acid increases the reduction rate

5 Changing the pH from 6 7 to 7 8 has little effect on the reduction rates with the exception of alcohol which reduces more rapidly at the higher pH

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STUDIES IN THE ABSORPTION DISTRIBUTION AND ELIMINATION OF ALCOHOL

VIII The Diuresis from Alcohol and Its Influence on the Elimination of Alcohol in the Urine

HOWARD W HAGGARD LEON A GREENBERG and RICHARD P CARROLL

From the Laboratory of Applied Physiology Yale University and the Connecticut Department of State Police

Received for publication January 16 1941

A number of investigators have administered alcohol and determined the amount which is eliminated in the urine the values found range from 0 7 to 4.3 per cent (1–10) These values do not express any general rate of elimination they are significant only for the particular experiments performed No study has heretofore been made of the general factors determining the rate of elimination of alcohol in the urine

The principles of elimination are defined here and demonstrated experimentally They also apply to the elimination of volatile substances other than alcohol Especial consideration is given to the diuresis caused by alcohol and its influence on the rate of elimination

1 PRINCIPLES OF ELIMINATION IN THE URINE

In the kidneys, alcohol passes from the blood into the urine by diffusion (10 11) The concentration of alcohol in the two fluids is determined by the relative solubilities of alcohol in the two media. This ratio varies slightly with the specific gravity of the urine for human urine at sp. gr 1 028 the ratio (with blood taken as 1) is 1.27 at sp gr 1 012, 1.30 and at sp gr 1 002, 1.34 (12)

The blood, in passing through the kidneys, loses alcohol to the urine The concentration in the urine does not, therefore correspond exactly to that in the arterial blood, but instead to that in the renal venous blood The loss of alcohol is so small however that no serious error is introduced in employing the concentration in the arterial blood

The relation of the concentration of alcohol in the arterial and venous bloods of the kidneys is shown by the equation,

$$(1) \qquad c \quad c' \quad V \quad V + kU$$

in which c and c' are respectively the concentrations in the renal arterial and venous bloods in milligrams per cubic centimeter V the volume of blood in cubic centimeters flowing through the kidneys per minute U the volume of urine in cubic centimeters secreted in 1 minute and k the solubility of alcohol in urine with that in blood taken as 1

The volume of blood flowing through the human kidneys per minute usually exceeds 1,000 cc the average reported by Smith Goldring and Chassis (13, 14) for 43 subjects was 1 180 cc. the lowest value was 525 cc The normal average output of urine by our subjects was 0 62 cc per minute the greatest output during the diuresis from alcohol

was 12 cc per minute On the basis of these values (equation *1*), the concentration of alcohol in the venous blood of the kidneys would, on the average, differ from that in the arterial blood by only 0 08 per cent and in the extreme, 2 9 per cent

The amount of alcohol eliminated by the kidneys during any period of time when the rate of secretion, blood flow, and concentration are uniform, is expressed by the equation

$$A = c'kV' \tag{2}$$

in which A is the amount of alcohol in milligrams eliminated in 1 minute, and c', k and V' are as in equation *1*

The amount of alcohol ingested is a primary factor in determining the amount of alcohol eliminated The relation, however, is not one of direct proportion The maximum concentration of alcohol reached in the blood, and the length of time required for the oxidation and elimination of the alcohol, both vary in approximate proportion to the amount of alcohol ingested That is, on doubling the dose the concentration will rise nearly twice as high and alcohol will remain in the body nearly twice as long (see table 1) Therefore with a uniform rate of secretion of urine, the *amount* of alcohol eliminated in the urine (equation *2*), will vary as the square of the amount ingested, while the *per cent* of alcohol eliminated will vary in direct proportion to the amount ingested

In most individuals alcohol causes a short period of diuresis at the time when the concentration of alcohol in the blood is highest, which is also when the urine contains the greatest amount of alcohol This diuresis interferes with the experimental demonstration of the principles of elimination defined here Two out of 12 subjects used in experiments to be reported here gave no diuresis with amounts of alcohol up to 48 grams These 2 subjects were therefore used in a series of experiments to determine the relation of the amount of alcohol ingested to that eliminated The findings are given in section 3 and table 1

2 GENERAL EXPERIMENTAL PROCEDURE

Twelve male subjects were used, they varied in age from 26 to 52 years and in weight from 62 to 91 kgm They came to the laboratory in the morning without having eaten since the evening meal of the previous day or taken water since 10 p m They remained seated in a comfortably warm room, reading or listening to radio music, and were kept as free as possible from disturbing influences A preliminary series of 4 experiments was carried out on each subject to determine the normal rate of urinary secretion For a period of 6 hours, during which no fluid was taken, the urine was collected and measured each 30 minutes In a second series of 4 experiments on each subject the urine was similarly collected for 2 hours before and for 6 hours after drinking 180 cc of water In all subsequent experiments in which alcohol was given it was diluted to this volume which, with a dose of 46 grams of alcohol, gave the highest concentration which the subjects could drink without discomfort The volume of water did not alter the hourly rate of urinary secretion beyond the limits of normal variation Figure 1 shows values obtained from one subject The average rate of secretion varied widely as between the different subjects, ranging from 24 to 74 cc per hour, but was remarkably constant for each subject From the 8 preliminary experiments the average hourly secretion was obtained for each subject, the maximum difference found between this average and that from a single experiment on any one subject was +9 and −7 cc per hour

In all experiments in which the elimination of alcohol was followed, the urine was collected at 30 minute intervals starting 2 hours prior to the ingestion of the alcohol and continuing for 1 hour after the alcohol had disappeared from the blood The 2 hour preliminary period was intended to detect any wide variation from the normal average of the subject, none was found The concentration of alcohol in each specimen of urine was determined and likewise, at the half-hour intervals, that in the blood

Diuresis occurring after drinking alcohol was recorded as the amount of urine secreted for the total period in excess of the average amount secreted by the subject in the same length of time when no alcohol but only water was drunk. After 12 grams of alcohol the urine was collected for 3 hours, after 24 grams for 5 hours after 48 grams for 9 hours and after 64 grams for 10 hours With a maximum individual variation from the average of 9 cc per hour as found in the preliminary experiments the volumes recorded here as diuresis may be taken for the time employed as significant when in excess respectively of 27 45 81 and 90 cc.

Völtz Baudrexel and Dietrich (15) and Nicloux and Nowicka (16) have shown that alcohol can be absorbed from the bladder Their demonstrations were made with alcohol injected into the bladder at concentrations higher than those found in the urine

TABLE 1

	URINE, AVERAGE	MAXIMUM CONCENTRATION ALCOHOL IN BLOOD	TIME ALCOHOL IN BLOOD	ALCOHOL ELIMINATED IN URINE	
				Amount	Per cent
	cc. per hour	mgm./cc.	hours	mgm.	
No water	26				
	28				
	31				
	27				
180 cc water	25				
	27				
	32				
	24				
12 grams alcohol diluted 180 cc	27	0 24	2	7 2	0 06
	29	0 24	2	7 9	0 07
24 grams alcohol diluted 180 cc.	29	0 52	3½	26 4	0 11
	30	0 54	3½	29 9	0 13
48 grams alcohol diluted 180 cc	31	0 92	7	115 2	0 24
	34	0 97	7½	121 8	0 26

after the ingestion of alcohol. Haggard, Greenberg, Carroll and Miller (12) have shown that for concentrations within the physiological range this absorption is insignificant The fact that in the experiments reported here the urine was collected at half-hour intervals instead of being permitted to accumulate until the subjects felt a desire to urinate in no way affects the amount eliminated (12)

3 ELIMINATION OF ALCOHOL IN RELATION TO AMOUNT INGESTED

Table 1 gives the results obtained on 1 of the 2 subjects who showed no diuresis after drinking 12, 24 and 48 grams of alcohol The corresponding maximum concentrations of alcohol reached in the blood (col 3) and the times required for the alcohol to disappear from the blood (col. 4) are approximately proportional to the amount of alcohol ingested. The per cent of alcohol eliminated (col. 6) varies approximately in proportion to the amount given

the total amount eliminated (col 5) varies approximately as the square of the amount given The per cents of alcohol eliminated as found here are lower than those previously reported in the literature, and also elsewhere in this paper, for the reason that with this subject no diuresis occurred

4 THE DIURESIS FROM ALCOHOL

Mendel and Hilditch (17) in 1910, on the basis of experiments carried out on 2 men given alcohol, concluded that alcohol did not cause diuresis and that the diuresis frequently observed after drinking alcoholic beverages was due *to ingredients other than alcohol* Their conclusions have been extensively quoted (18) in spite of the fact that numerous investigators (8, 20, 21, 22) since that time, most recently Bruger, Localino and Guthrie (22), have shown that alcohol alone may cause diuresis, not only in normal human subjects, but also in those with renal disease We find, however, that there is a marked individual difference in the extent of the diuresis, and that for some men diuresis occurs only after large amounts of alcohol This fact offers a possible explanation for the failure of Mendel and Hilditch to obtain a diuresis when using only 2 subjects

Table 2 gives the results obtained from 84 experiments on 12 subjects given 12, 24, 48 and 64 grams of alcohol

After 12 grams of alcohol, 9 of the 12 subjects showed a definite diuresis ranging from 31 to 120 cc , after 24 and 48 grams of alcohol, 10 out of the 12 subjects gave a diuresis ranging from 48 to 219 cc for the smaller dose and from 121 to 542 for the larger After 64 grams all subjects showed diuresis ranging from 136 to 727 cc

The extent of diuresis for any subject had a general, but not invariable, relation to the normal rate of urinary secretion (col 2), those subjects who had the highest normal rates usually had also the greatest diuresis In general, the diuresis increased with the amount of alcohol given, but here also there were exceptions Thus subject 4 had no greater diuresis with 64 grams of alcohol than with 24 Although there were wide variations between the diuresis in different subjects, the diuresis of individual subjects was quite constant for repeated experiments

The fact that alcohol causes diuresis does not remove the possibility that the essential oils and other ingredients of alcoholic beverages may also cause diuresis It is widely held that gin, because of its content of oil of juniper berries, has a particularly marked diuretic action (24) There are also differences in the rate of absorption of the alcohol from different beverages with corresponding differences in the maximum concentration of alcohol reached in the blood (24)

The comparative diuresis caused by alcohol, gin, whisky, port, and sherry wines, was determined on 4 subjects (1, 3, 5 and 6 of table 2) For each beverage the total alcohol given was 24 grams, the volume as needed was brought to 180 cc with water With alcohol the average diuresis for the 4

subjects was 121 cc with American gin 85 cc., Holland gin 109 cc , whisky, 116 cc sherry wine 62 cc. and port wine 53 cc None of the beverages gave

TABLE 2

SUBJECT	AGE AND WEIGHT	NORMAL HOURLY SECRETION OF URINE	AMOUNT OF ALCOHOL GIVEN IN 180 CC. FLUID							
			12 g.		24 g.		48 g		64 g.	
			Diuresis	Eliminated in urine	Diuresis	Eliminated in urine	Diuresis	Eliminated in urine	Diuresis	Eliminated in urine
		cc.	cc.	per cent	cc.	per cent	cc.	per cent	cc.	per cent
1	48 years 75 kgm.	27	0 4	0 06 0 07	8 12	0 11 0 13	28 51	0 24 0 25	186	0 78
2	52 years 71 kgm.	23	0 19	0 08 0 08	7 11	0 13 0 09	31 53	0 27 0 24	210	0 89
3	45 years 78 kgm	26	15 21	0 10 0 11	48 51	0 24 0 25	121 184	0 63 0 78	378	1 42
4	39 years 73 kgm	30	31 37	0 11 0 16	137 182	0 32 0 37	207 267	0 84 0 83	408	1 54
5	29 years 76 kgm	33	20 57	0 14 0 17	166 219	0 65 0 82	360 468	1 09 1 15	465	1 65
6	37 years 62 kgm	34	66 91	0 16 0 19	137 164	0 50 0 68	182 197	0 92 1 03	252	0 86
7	32 years 73 kgm	4[illegible]	60 83	0 16 0 21	188 164	0 39 0 53	182 147	0 84 0 97	138	0 82
8	46 years 79 kgm	42	21 33	0 12 0 16	82 163	0 47 0 53	171 177	0 99 1 14	328	1 62
9	53 years 84 kgm	45	90 110	0 21 0 24	147 164	0 31 0 30	293 354	1 01 1 19	491	1 92
10	32 years 76 kgm	52	12 40	0 16 0 15	82 163	0 28 0 41	215 273	0 82 0 09	401	1 81
11	26 years 77 kgm	67	98 120	0 24 0 40	130 148	0 44 0 61	320 542	1 20 1 30	727	3 54
12	31 years 84 kgm.	74	90 102	0 22 0 29	178 212	0 51 0 58	199 207	0 87 1 04	514	3 14

a greater diuresis than that expected from the alcohol content and the wines gave definitely less.

It was impossible to use beer in this series of experiments since 180 cc

yield only about 6 grams of alcohol Therefore, 720 cc containing 24 grams of alcohol were given and the rate of secretion of urine compared with that after the same amount of water In 2 experiments on subject 4, the amounts of urine secreted during 5 hours after water were 560 and 579 cc , and after beer, 689 and 721 cc The corresponding values for subject 5 were 692 and 765 cc and 853 and 951 cc It would appear that, aside from its bulk of water, beer caused no greater diuresis than would be expected from the content of alcohol (25)

When alcohol is taken with only small amounts of water, as in most of the experiments given here, the rate of urinary secretion following the diuresis falls below that which existed before the alcohol was taken In the experiment presented in figure 1 the average rate of secretion from the 3d to the 9th hour after water only was taken was 29 5 cc per hour, for the same period after alcohol was taken the secretion was 22 2 cc per hour In other experiments the decrease was more marked A similar decrease is seen in values given by Miles (8) and by Murray (27) from experiments in which the alcohol was given in 100 and 300 cc of fluid In the experiments of Miles in which the alcohol was given in 1,000 cc of fluid, and here after 720 cc of beer, the decrease below the preliminary level did not occur

5 THE INFLUENCE OF DIURESIS ON THE ELIMINATION OF ALCOHOL

The amount of urine secreted, as Haggard and Greenberg (12) have previously shown, and as is further demonstrated here (table 2), is a controlling factor in the elimination of alcohol Thus after 48 grams of alcohol, subject 1 had, during 11 hours, a total secretion of 433 cc which contained 0 362 grams of alcohol (0 78 per cent of that ingested), under the same conditions, subject 6 had a total secretion of 1464 cc containing 1 760 grams of alcohol (3 84 per cent of that ingested) The amounts of alcohol eliminated by these two subjects are not, as would be expected from equation 1, proportional to the amounts of urine, instead, for unit volumes of urine the amounts of alcohol eliminated are in the relation of 1 1 2 The concentrations in the blood were nearly identical in these two experiments The disproportion in the amounts eliminated results from the fact that the diuresis from alcohol occurs during the time when the concentration of alcohol in the blood is at or near its maximum, the concentration of alcohol in the urine is therefore highest at the time when the greatest amount is secreted In consequence the major portion of the alcohol eliminated appears in the urine within less than half the time the alcohol remains in the blood, this fact has been previously observed (8) The relations involved are illustrated in figure 1 which gives the full data for the experiments in which subject 4 was given 64 grams of alcohol

6 THE CONCENTRATION OF ALCOHOL IN THE BLOOD IN RELATION TO DIURESIS

The diuresis from alcohol commences within a half-hour after the alcohol is ingested and continues as long as the concentration in the blood is rising

Unless complicated by diuresis from large amounts of water taken with the alcohol, the diuresis from alcohol subsides rapidly after the maximum concentration of alcohol in the blood is reached (see Fig 1) The diuresis does not depend alone upon the presence of alcohol in the blood a high concentra-

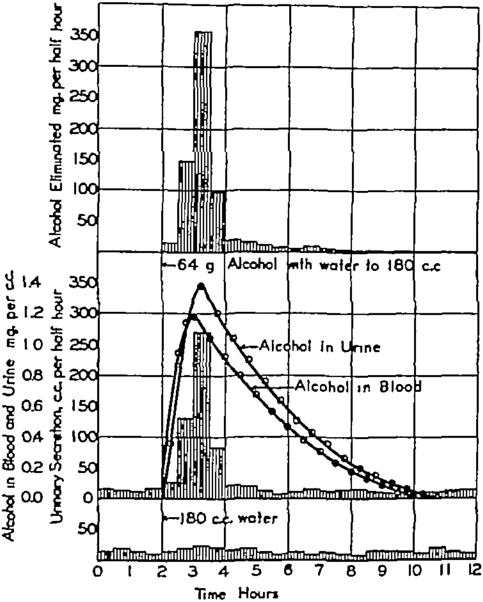

Fig 1

tion which is not rising may exist in the blood without exciting diuresis. *Thus in the experiments presented as figure 1 the concentration of alcohol* in the blood following the completion of diuresis was much higher than the maximum concentration reached in other experiments (table 2) in which smaller amounts of alcohol were given and were followed by diuresis Although the diuresis ceases when the concentration passes its maximum and

starts to decline, it can again be excited if more alcohol is given with further rise in the concentration as was demonstrated by the following experiment Subject 5 took 24 grams of alcohol in a total of 90 cc of fluid The rate of urinary secretion preceding the ingestion of the alcohol was 33 cc per hour, *during the 1½ hours after the alcohol was taken, there was a diuresis of 147 cc* , and during the next 1½ hours the rate of secretion fell to 24 cc per hour The same amount of alcohol was then taken in a total of 90 cc of fluid A second diuresis of 163 cc occurred in the following 1½ hours

Murray (21) has concluded that the diuresis from alcohol results from the depression of the pituitary antidiuretic element as in water diuresis The fact that the diuresis occurs only during the time that alcohol is present in the alimentary tract and is being actively absorbed, i e , rising concentration in the blood, suggests the possibility that the action may be exercised through reflexes from this tract This possibility, however, was disproved by an experiment in which alcohol was administered intravenously Twenty-four grams of alcohol, diluted to 180 cc with water, were first given by stomach, the resulting diuresis was 387 cc Two days later the same amount of alcohol, diluted to 180 cc with saline, was injected intravenously at a uniform rate during 30 minutes, the resulting diuresis was 337 cc No diuresis developed when 2 days later 180 cc of saline without alcohol were similarly injected

CONCLUSIONS

1 At a uniform rate of urinary secretion, the amount of alcohol eliminated in the urine varies approximately as the square of the amount ingested, the per cent eliminated varies directly with the amount ingested

2 The greatest variation in the amount eliminated results from the diuresis caused by alcohol

3 There are wide individual differences in the extent of diuresis from alcohol

4 Ingredients of alcoholic beverages other than alcohol do not contribute to the diuresis

5 Diuresis occurs only when the concentration of alcohol in the blood is rising It does not result from the mere presence of alcohol in the blood and *does not occur when the concentration is stationary or falling*

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STUDIES ON THE ABSORPTION, DISTRIBUTION AND ELIMINATION OF ALCOHOL

IX The Concentration of Alcohol in the Blood Causing Primary Cardiac Failure

H W HAGGARD, L A GREENBERG, L H COHEN AND N RAKIETEN

From the Laboratory of Applied Physiology, Yale University

Received for publication January 16, 1941

In a study previously presented in this series of papers (1) we have determined the concentration of alcohol in the blood causing respiratory failure For normal fasted rats the average of the concentrations found was 9 31 mgm per cubic centimeter, the extremes, 8 9 and 10 0 mgm per cubic centimeter The present paper reports an extension of this study in the determination of the concentration in the blood causing cardiac failure

In death from acute poisoning from alcohol cardiac failure is ordinarily secondary to respiratory failure The action of the heart is affected, but not seriously impaired, by the concentration of alcohol in the blood causing respiratory failure The heart continues to beat for a short time after breathing has stopped, but it rapidly succumbs to anoxemia In the present study anoxemia was prevented by artificial respiration, the administration of alcohol was continued, after spontaneous breathing had stopped, until the concentration had risen sufficiently to cause cardiac failure

Young male rats were used, they were fasted 16 hours (1) No description could be found, in the literature, for an apparatus for maintaining prolonged artificial respiration in this animal The apparatus, shown in detail in figure 1, was developed for this purpose Although no new principle is involved, considerable experimentation was necessary in the adaptation of the apparatus to the small animal

In determining the concentration of alcohol causing cardiac failure it is imperative that the volume of lung ventilation be adjusted precisely to the needs of the animal The anoxemia from insufficient ventilation, and the acapnia from excessive ventilation, both cause premature failure The necessary adjustment was made before respiratory failure had occurred When the animal was fully unconscious from alcohol, but still breathing the trachea was exposed and slit for insertion of the cannula This cannula fitted snugly into the trachea, but was not tied in place Artificial respiration was carried out for 3 minutes, after which the cannula was removed and the breathing of the animal observed If apnea occurred, the pressure of the air or the speed of the motor, or both were reduced slightly and if no apnea occurred, they were increased After a suitable interval, artificial respiration was again carried out for 3 minutes and the subsequent breathing observed These procedures were repeated until the ventilation was finally adjusted to a volume slightly less than that which produced apnea

If correctly adjusted no apnea developed after artificial respiration had been applied for 15 minutes and likewise during this period no attempt was made by the animal to breathe out of rhythm with the artificial respiration The cannula was then removed, but the apparatus kept in operation so that artificial respiration could be started when respiratory failure from the alcohol was imminent

Up to the time of respiratory failure the alcohol was administered at the slow rate which we have employed in determining the concentration at respiratory failure (1) thereafter the rate was increased from 0 125 mgm per gram of body weight each 5 minutes to 0.25 mgm In determining the concentration causing cardiac failure it is

FIG 1 APPARATUS FOR ARTIFICIAL RESPIRATION IN RATS

Flow of air is regulated by needle valves *A* and *B* Rotor of rotating cock *D* is 1 cm in diameter is ground into stationary barrel and is lubricated with film of petrolatum Speed of rotation regulated to give 30 to 40 respirations per minute Pressure as read from manometer *D* 6 to 10 cm. of water during inspiration 0 during expiration

not necessary to employ the extremely slow rate essential in determining the concentration causing respiratory failure It is necessary only that there shall be a steadily rising concentration of alcohol in the blood with no wide fluctuations Respiratory failure from alcohol results from the action of the alcohol on the respiratory center the concentration of alcohol in the center can be inferred from that in the arterial blood only when the concentration in the venous blood leaving the brain corresponds closely to that in the arterial blood (1) This correspondence can be maintained only by a very slow rate of administration On the contrary the lethal action of alcohol on the heart appears to be exercised directly upon the heart muscle This fact has been shown by experiments on the perfused heart (2-8) It is supported here by two additional observations

In three experiments it was found that cutting the vagi did not alter the concentration

of alcohol causing cardiac failure Further, in numerous experiments in which alcohol, in a concentration of 50 per cent by volume, was administered rapidly into the jugular vein, we have been able to produce primary cardiac failure with respiration continuing for 20 to 30 seconds as after ventricular fibrillation from electric shock Under these unusual conditions the concentrations found in the blood in the heart equaled or exceeded those reported here as causing cardiac failure, but the concentrations obtained from the remaining jugular vein, expressing the concentrations effective in the brain, were lower than those causing respiratory failure In these experiments, the heart was affected directly and immediately by the high concentration of alcohol in the blood reaching it, it ceased beating before sufficient alcohol had been circulated to the brain to build up a high concentration there We have previously reported similar experiments (1), as have other investigators (9)

When, under slow administration of alcohol as employed here, the concentration in the blood approaches that causing respiratory failure, a moderate bradycardia develops After breathing fails, if artificial respiration is not

TABLE 1

Concentration of alcohol in heart blood at cardiac failure

RAT NUMBER	MGM /CC	RAT NUMBER	MGM /CC	RAT NUMBER	MGM /CC
1	12 1	9	12 9	17	12 8
2	12 9	10	12 3	18	12 3
3	12 2	11	13 1	19	12 5
4	13 0	12	12 4	20	12 7
5	12 8	13	12 5	21	13 2
6	12 1	14	12 0	22	12 8
7	12 9	15	12 9	23	12 8
8	12 7	16	12 6	24	12 6

started the further alterations in cardiac function are entirely those from anoxemia There is partial, and usually complete, block with failure often in ventricular fibrillation When, as here, artificial respiration is instituted as spontaneous breathing fails, the heart continues to beat with apparently normal action, as judged by the heart sounds, until the concentration has risen nearly 30 per cent higher than that causing respiratory failure The toxic action of the alcohol on the myocardium then becomes clearly evident When the chest is opened and the heart observed, the ventricles appear relaxed and the contractions weak (4, 5, 8) Auricular contractions continue, but auriculo-ventricular conduction is impaired and partial block develops The ventricular beat becomes irregular and then stops in diastole with marked dilatation

Table 1 gives the concentration of alcohol found in the blood for 24 experiments The average concentration is 12 6 mgm per cubic centimeter, the extremes, 12 0 and 13 2 mgm per cubic centimeter

The considerable difference between the concentrations of alcohol causing

respiratory failure and cardiac failure suggests the possibility of resuscitation in acute poisoning from alcohol with respiratory failure In three experiments alcohol was given until respiratory failure occurred, the administration was then stopped and artificial respiration started immediately It was continued for one hour, at this time the concentration in the blood had fallen below that causing respiratory failure. On stopping artificial respiration spontaneous breathing occurred in all three animals it persisted in two but failed in one, even after a second period of artificial respiration.

CONCLUSIONS

1 Cardiac failure in acute poisoning from alcohol results from anoxemia following respiratory failure

2 The heart is not seriously injured by the concentration of alcohol which causes respiratory failure.

3 For rats in which anoxemia was prevented by artificial respiration the concentration of alcohol in the blood causing cardiac failure was found to average 12.6 mgm. per cubic centimeter with extremes of 12 0 and 13.2 mgm. per cubic centimeter

4 The main toxic action of the alcohol is exercised upon the myocardium.

5 Following acute poisoning from alcohol cardiac failure can be prevented by employing artificial respiration and spontaneous breathing may be restored.

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THE ELECTROCARDIOGRAM IN ACUTE EMETINE INTOXICATION[1]

LINN J BOYD, M D, F A C P, AND DAVID SCHERF, M D

From the Department of Pharmacology, New York Medical College, Flower and Fifth Avenue Hospitals

Received for publication February 13, 1941

It has been known for a long time that emetine may adversely affect the circulatory system Although fall of blood pressure, cardiac dilatation, and profound morphological changes in the myocardium have been observed repeatedly, knowledge of the electrocardiographic alterations in emetine poisoning is very limited Only a few incomplete and, to some extent, contradictory studies on this subject have been published The experiments reported in this communication were designed to examine the electrocardiographic aspects of acute emetine intoxication

The experiments were performed on 33 dogs and 4 cats The animals were anesthetized by the intraperitoneal injection of nembutal (0 05 grams per kilogram body weight) In all experiments the sternum was removed and the pericardium opened in order to permit direct observation of the heart The electrocardiogram was recorded in the three conventional leads Emetine hydrochloride (Lilly) was injected into the femoral or jugular vein The dogs for these experiments came from the same stock but varied between 5 and 9 kilograms in weight In six experiments the vagus was divided prior to the administration of the drug without changing the results in any respect

Statements concerning the lethal dose of emetine hydrochloride after a single intravenous injection vary considerably even when based upon experience with the same species of animal Although some investigators (1, 12, 14) report 4 to 18 milligrams per kilogram body weight as lethal under these conditions, others (18) find 40 to 80 milligrams per kilogram necessary In our experiments 37 milligrams per kilogram (½ grain) was the largest amount from which animals regularly recovered after intravenous injection Greater amounts frequently caused cardiac death in a few minutes

RESULTS

(a) *Disturbances of intraventricular conduction*

The earliest and most striking alteration of the electrocardiogram after the injection of emetine hydrochloride consists of a widening of the initial complex This occurs as early as 20 to 30 seconds after the injection and is not accompanied by a decided prolongation of atrioventricular conduction time, by an unusual change of rate, or by marked alteration of the T-wave

[1] Aided by a grant from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association

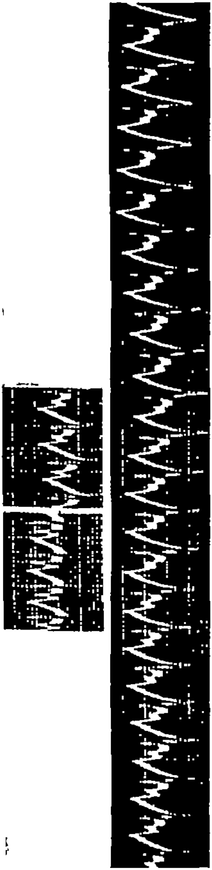

Fig 1 Dog before (upper left tracing) and after (upper right tracing) the injection of 37 mgm. of emetine hydrochloride The lower tracing, recorded a few seconds later shows the gradual increase of width of the initial complex

The result of a typical experiment may be described briefly, the tracing is reproduced in figure 1 After the division of both vagi there was a sinus tachycardia with a rate of 222 (left upper tracing) Twenty-two seconds after the injection of 37 mgm of emetine hydrochloride into the jugular vein the S-wave became deeper and the initial deflection slightly wider (right upper tracing) The rate fell to 200 An electrocardiogram recorded four seconds later (lower tracing) shows the initial deflection rapidly increasing in width to 0 16 second The S-wave is almost exclusively responsible for the greater width of the initial deflection The T-waves are unaffected, the rate has fallen to 157 and the conduction time, which was 0 09 second before the injection, becomes 0 11 second after it

If no disturbance of rhythm developed the bradycardia increased for about 90 seconds while the width of the initial complex remained fixed The disturbance of intraventricular conduction and bradycardia gradually disappeared so that ultimately, after 45 minutes at the most, the electrocardiogram resumed the same appearance as prior to the injection

The changes just described were obtained in all experiments The R-wave, ordinarily small in Lead I, became broader and it was typical for a deep S-wave to appear and rapidly to increase in width in Leads II and III The T-wave remained unchanged or became positive if, as is often the case in dogs, it was previously negative

These findings are demonstrated by the reproduction of tracings (fig 2) from a dog weighing 9 kgm The three standard leads are normal before the injection (fig 2a) One minute after the injection of 37 mgm of emetine hydrochloride (fig 2b) the width of the initial deflection has increased from 0 05 second to 0 12 second and a deeper S-wave has appeared, in this experiment the R-wave in Leads II and III also exhibited widening although this was not the rule The heart rate fell from 145 to 122 and the auriculo-ventricular conduction time was prolonged from 0 11 to 0 13 second The T-wave has become higher Twelve minutes later (fig 2c) the alterations have begun to recede The rate is increased to 132, the S-wave is shorter, and the initial complex is only 0 09 second in width Twenty-seven minutes after the original injection of emetine the electrocardiogram is normal (fig 2d)

The second intravenous injection of an equal amount of emetine hydrochloride produced similar alterations although they were more distinct (fig 2e), the rate fell to 100, the initial complex became 0 16 second in width One minute later (fig 2f) the initial complex became wider (0 20 second) and auricular extrasystoles appeared The P-wave of the auricular extrasystole is superimposed on the T-wave of the preceding beat The alterations also receded after this injection so that an electrocardiogram, practically normal in configuration, was recorded twenty-five minutes after the second injection (fig 2g)

In this experiment a third intravenous injection of 37 mgm of emetine

hydrochloride was given Grouped auricular extrasystoles and widening of the initial complex appeared as in the two preceding injections a few minutes

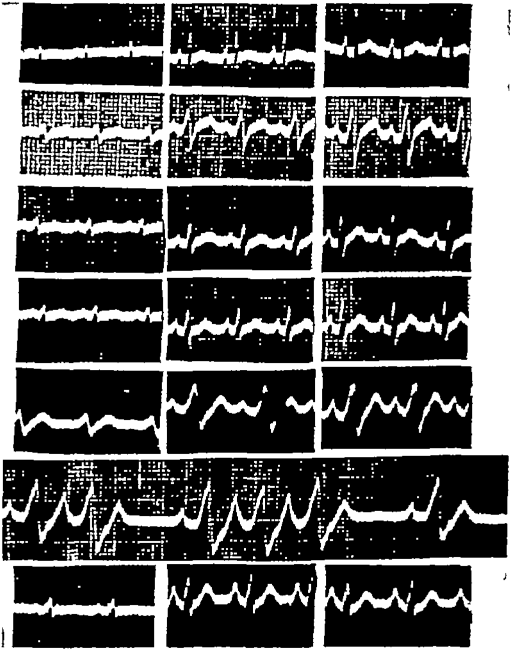

Fig 2 Dog, fig 2a shows the electrocardiogram before and fig 2b immediately after the injection of 37 mgm of emetine figs 2c and 2d were taken 12 and 27 minutes later respectively fig 2e was obtained after a second injection of emetine which produced extrasystoles in one minute (2f) fig 2g shows the electrocardiogram 25 minutes after the second injection

later marked bradycardia followed by diastolic standstill of the heart were observed

In this experiment as in all others the second or third injection of equal

amounts of emetine hydrochloride had much more pronounced effects than the first, despite the complete disappearance of the alterations of the electrocardiogram evoked by the preceding injections The third injection usually caused cardiac standstill Whenever the initial complex exceeded 0 20 second in width, recovery did not occur

(b) Alterations of cardiac rhythm

Auricular extrasystoles were common (fig 2) and, at times, were suddenly converted into paroxysmal auricular tachycardias When this happened confusing pictures occasionally developed since a disturbance of intraventricular conduction might co-exist

Figure 3 shows the electrocardiogram of a dog weighing 6 6 kgm , it was normal before the injection of emetine (first electrocardiogram in upper series

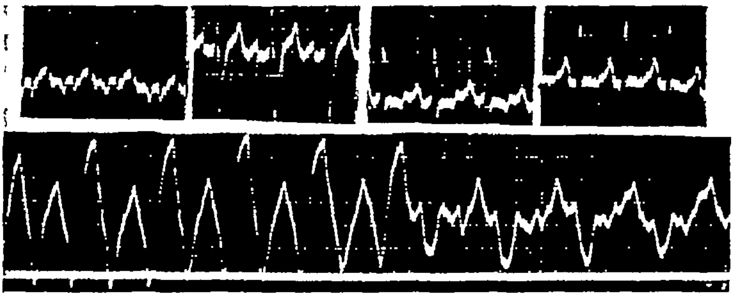

FIG 3 Dog, lead III The first upper tracing at the right shows the electrocardiogram before, the second after the injection of 37 mgm of emetine The third and fourth tracings were taken after 10 and 20 minutes respectively and show recovery The lower tracing reproduces a tachycardia with a disturbance (alternans) of the intraventricular conduction after repetition of an equal amount of the drug

of figure 3, Lead III is shown) The P- and T-waves are negative and the initial deflections are so thin that they are almost invisible After the injection of 37 mgm of emetine hydrochloride (second tracing) the S-wave widens in a typical manner and the P- and T-waves become positive The cardiac rate fell from 166 to 142, while the auriculo-ventricular conduction time increased from 0 12 to 0 16 second The other tracings in the upper series of figure 3, show the same lead after the lapse of ten and twenty minutes respectively Definite recovery occurred, the rate increased, the width of the initial complex decreased, and the T-wave again tends to become negative Repetition of the intravenous injection (37 mgm) produces a tachycardia in a few seconds (fig 3, lower tracing) At first glance a ventricular tachycardia seems to be present but more careful analysis shows a constant auricular tachycardia with a rate of 200 The P-waves are hidden in the preceding ventricular complexes, the latter are markedly widened as the result of a

disturbance of intraventricular conduction Owing to the presence of a conduction alternans the T waves vary in height In the middle of this tracing the picture suddenly changes There is a decided increase of alternation of intraventricular conduction presumably the result of progressive toxic action so that one ventricular complex with a deep S-wave alternates

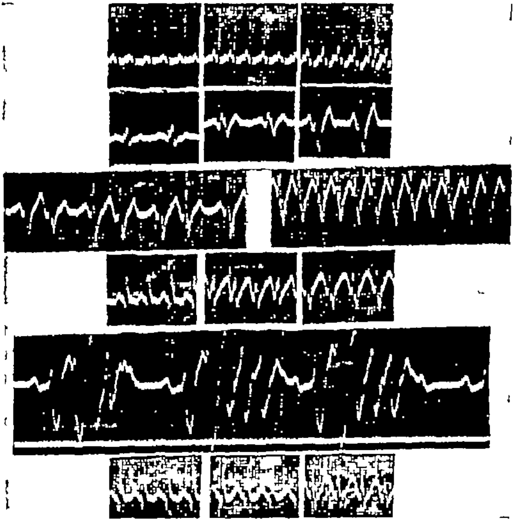

FIG 4 Dog fig 4a was taken before 4b after the injection of 37 mgm of emetine hydrochloride Fig. 4c shows auricular extrasystoles and paroxysmal auricular tachycardia one minute after the injection Fig 4d was obtained 30 minutes later fig 4e was recorded after a second injection of equal amounts of the drug and fig 4f shows the status 45 minutes later

with another which is almost isoelectric but exhibiting a very high T wave. This type of alternating conduction was not uncommon (See fig 6a)

Another variety of disturbance of rhythm is shown in figure 4 The first tracing (fig 4a) shows a sinus tachycardia in a dog weighing 6 2 kgm after the division of the vagi and before the administration of emetine hydrochloride The next tracing (fig 4b) shows the effect of an intravenous in

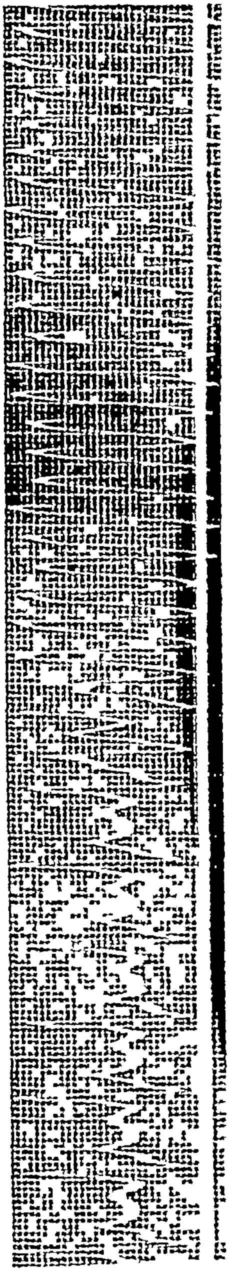

Fig 5 Dog, lead II Paroxysmal ventricular tachycardia after emetine

jection of 37 mgm. of the drug The widening of the initial complex, affecting the S-waves in particular is again evident in Lead III One minute after the injection auricular bigeminy (fig 4c) appeared and was rapidly converted into a tachycardia. Since the shape of the ventricular complex remains unchanged an auricular tachycardia presumably is present The alternating form of ventricular complexes is also apparent The lapse of thirty minutes permitted great improvement to occur (fig 4d) but recovery was not complete Repetition of the same dose of the drug produced more pronounced effects. The conduction time is prolonged to 0 26 second the initial deflection becomes 0 20 second in width (fig 4e) and abnormal ventricular complexes of variform appearance become frequent Probably they represent ventricular extrasystoles but auricular extrasystoles with abnormal

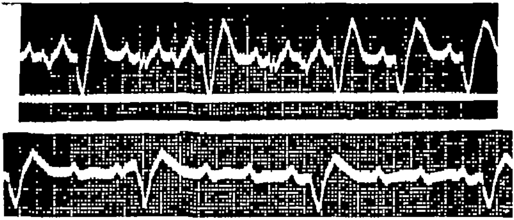

FIG 6 Cat upper tracing shows an auricular tachycardia with disturbances of auriculo-ventricular and intraventricular conduction after 37 mgm emetine hydrochloride Lower tracing shows 2 1 and 4 1 auriculo-ventricular block

intraventricular conduction cannot be excluded Forty five minutes later *recovery was approximately complete (lowest tracings fig 4f)*

Ventricular tachycardias were less common than auricular tachycardias Although the former type appeared late in the intoxication in some experiments complete recovery so far as the electrocardiogram was concerned, was possible despite their development Ventricular tachycardia is shown in figure 5 This tachycardia the rate approximating 300 per minute began after a second injection of 37 mgm. of emetine hydrochloride a period of 22 minutes elapsing between the two injections Ventricular tachycardias of this type frequently change into ventricular fibrillation Auricular fibrillation was encountered only twice in the entire group of experiments

Apart from relatively slight prolongation of auriculo-ventricular conduction time no other disturbance of conduction between auricle and ventricle occurred in dogs However complete heart block was noted in two cat experiments A cat weighing 4 7 kgm received 37 mgm of emetine hydrochloride intravenously (fig 6) Subsequently an auricular tachycardia

with a rate of 166 appeared (upper tracing) The first auricular beat after the beginning of the tracing is conducted slowly and is followed by a very small, narrow ventricular complex The second P-wave, concealed in the first beat, is followed by a ventricular complex whose appearance is entirely different than the first, the third P-wave is blocked Then come two groups of 4 3 block, similarly arranged and with variable aberrant ventricular complexes, at the end of the tracing there is 2 1 block in which the ventricular complexes present no differences in configuration Two minutes later 3 1 and 4 1 auriculo-ventricular block interchange (lower tracing) Shortly thereafter auriculo-ventricular conduction as well as ventricular automatism fail completely while the auricles continued to beat regularly for some time

Inspection of the heart during the action of the drug reveals marked cardiac dilatation affecting the right ventricle in particular This observation has also been made by Chopra The amount of dilatation roughly parallels the extent of widening of the ventricular complex and both changes gradually and simultaneously vanish

When acutely lethal doses of emetine are employed ventricular fibrillation develops in only 60 per cent of the experiments, in 40 per cent the automatism of the ventricle became progressively slower and finally failed with diastolic standstill of the heart Although automatism could be easily restored by the intracardiac injection of epinephrine, it rarely persisted

DISCUSSION

These experiments yielded uniform results The most obvious alterations after the injection of sub-lethal doses consisted of considerable widening of the ventricular complex accompanied by very definite cardiac dilatation Prolongation of auriculo-ventricular conduction time like slowing of the rate occurred but were moderate The T-waves in Leads II and III became positive if they were previously negative or remained positive if they originally had this form Since it was typical for the initial deflection to be directed downward in these leads, one may say, in general, that after emetine hydrochloride the T-waves tend to assume a reciprocal relation to the initial deflection

Auricular extrasystoles and auricular tachycardias were common arrhythmias in acute emetine intoxication Auricular fibrillation was rare, while ventricular extrasystoles and ventricular tachycardias were encountered, for the most part they were terminal and preceded ventricular fibrillation

These observations are not in agreement with the results obtained by some investigators who found a predominance of ventricular tachycardias and the frequent appearance of auricular fibrillation (3, 6) Some of the discrepancy disappears, however, when it is realized that the tracings which Bearman and Leake interpret as ventricular tachycardias represent auricular tachycardias with the disturbances of intraventricular conduction described above Chopra and Sen depict a tracing from a patient with emetine poison-

ing, it shows a definite depression of the S-T segment rather than the electrocardiographic findings we encountered in these experiments However no evidence was submitted to prove that the electrocardiographic alterations of that patient were the result of emetine therapy The appearance of disturbances *of conduction in the intact heart after large doses of emetine* was noted by Epstein.

Since emetine may be regarded as a general protoplasmic poison the widening of the initial complex is comprehensible as an expression of an injury of the specific tissue of the heart and a disturbance of the intraventricular spread of excitation Histological changes which prove the occurrence of myocardial damage have been repeatedly reported in man as well as animals after emetine poisoning (1, 5 18) Nevertheless it is striking that the electrocardiograms obtained from animals who have been subjected to *chronic* intoxications show very slight changes despite decided alteration of the histological picture (13)

The rapid recession of the disturbances demonstrable by the electrocardiograph simultaneous diminution of the cardiac dilatation, and the return of the strength of contraction, supplements older observations and makes them comprehensible Even in the early studies of the effect of emetine on the heart (9 17) the transient character of the fall of blood pressure after small doses of emetine (0 01 gram) was noted When this amount was repeated at short intervals death did not result a single injection of 0 02 gram of emetine, on the other hand produced immediate cardiac standstill with an abrupt fall of blood pressure. When dilute solutions were slowly injected the tolerated dose was much larger than with rapid injection of concentrated solutions (14) These reports and the experiments already described might suggest that emetine disappears rapidly from the blood or is rendered innocuous However such conclusions are at odds with the well known cumulative action of emetine first described by Dale likewise it is evident from the experiments reported that a second or third injection provokes progressively greater effects even when recovery of the electrocardiogram from the first injection seems complete.

Although no unequivocal report of an immediate fatality following the intravenous injection of emetine in man can be found nevertheless the experiments reported in this paper suggest that care should be exercised when large doses of emetine are given intravenously to man (2 13) Since the alterations which follow intravenous injection of the drug rapidly disappear there is reason for believing that these changes would not develop if an equal amount of emetine hydrochloride was subcutaneously administrated

CONCLUSIONS

The alterations of the electrocardiogram after the intravenous administration of emetine hydrochloride were studied in dogs and cats.

Disturbance of intraventricular conduction is the most common change

observed under these circumstances Bradycardia and prolongation of atrioventricular conduction time develop regularly but they are not pronounced

If the intravenous dose does not exceed 37 mgm, in dogs weighing 5 to 9 kgm the electrocardiographic alterations gradually disappear within 45 minutes Cardiac dilatation, especially involving the right ventricle, the development of which is simultaneous with the ventricular conduction disturbances, also vanishes within the same period

The action of emetine is cumulative since much more pronounced effects result from the second or third injection of an equal amount of the drug although the normal electrocardiogram had been restored

The most common arrhythmias to develop are auricular extrasystoles and auricular tachycardias Alternation of the ventricular complexes is frequently observed during the tachycardia Advanced stages of intoxication are required for the production of ventricular extrasystoles Heart block with dropped beats was encountered only in cats

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EFFECTS OF MORPHINE AND ITS DERIVATIVES ON INTERMEDIARY METABOLISM[1]

I THE INFLUENCE OF MORPHINE CODEINE AND THEBAINE ON THE ACTIVITY OF SEVERAL DEHYDROGENASES AND ON THE RESPIRATION OF RAT CEREBRUM

M H SEEVERS AND F E. SHIDEMAN

From the Department of Pharmacology University of Wisconsin, Madison

Received for Publication November 18 1940

The pharmacological actions of morphine and its derivatives have not been established on a biochemical basis. Numerous problems concerning the actions of these drugs, especially those related to chronic administration, would be nearer solution if such a basis could be established Relatively little attention has been devoted either to the effects of morphine and its derivatives on the oxidation of various substrates by isolated animal tissues or to its action on the oxidative enzymes, although numerous investigations have indicated that oxidative processes may be affected either directly or indirectly by the action of these drugs (1) According to Quastel and Wheatley (2) the extra oxygen uptake of rat brain mince in the presence of glucose and of lactic pyruvic and glutamic acids, is inhibited 30 per cent by 0 12 per cent morphine whereas no change occurs with the addition of succinic acid. Gross and Pierce (3) report a reduction in the oxygen uptake of rat brain mince in the presence of glucose when 0 12 per cent morphine was added but Wortis (4) could show no effect with either 0.008 or 0 032 per cent morphine Keeser (5) has studied *in vitro* the effect of morphine on a series of enzymes including tyrosinase urease reductase lecithinase phosphatase and serum lipase. Urease phosphatase and serum lipase activities were reduced by various concentrations of morphine whereas the activity of reductase from frog muscle was increased. Tyrosinase and lecithinase activity was either increased or decreased depending upon the concentration of morphine used. Keeser (6) also demonstrated an increased blood level of methyl guanidine following the administration of a single small dose of morphine this he attributed to an accelerating effect of morphine on lecithinase. Only two of the enzymes studied by Keeser are of a respiratory nature viz tyrosinase and reductase and of these only tyrosinase is specific

[1]Supported by the Wisconsin Alumni Research Foundation

The work presented in this paper is correlated with the program of the Committee on Drug Addiction of the National Research Council and allied agencies.

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cortex to determine whether or not the effects observed with the minced and the washed homogenized preparations were demonstrable with this type of surviving tissue

Results

The data in figure 1 represent the average oxygen uptake (U_{O_2} at two hours) of duplicate determinations made on various preparations of rat cerebrum

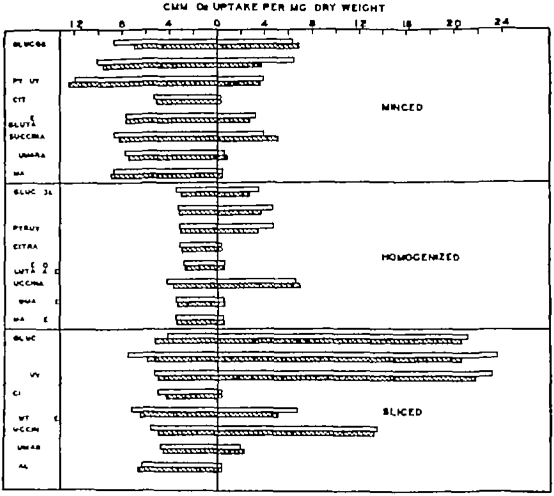

FIG 1 OXYGEN UPTAKE (U_{O_2} AT 2 HOURS) OF MINCED, WASHED HOMOGENIZED AND SLICED RAT CEREBRUM IN THE PRESENCE OF VARIOUS SUBSTRATES AND MORPHINE

Reading to left of zero line, unshaded bars represent the nosubstrate values the shaded bars no-substrate oxidation in presence of 0 12 per cent morphine To the right of zero line unshaded bars represent the extra oxygen uptake resulting from addition of substrate shown on left and shaded bars the substrate oxidation with added morphine

with and without added substrate and morphine The results presented for each substrate represent a characteristic experiment Since it was necessary for technical reasons to perform numerous experiments to determine the effects of morphine on the oxygen uptake of a given preparation in the presence of several substrates the values for the no-substrate oxygen uptake vary

quite widely from one experiment to the next. Each experiment involved the determination in duplicate of (*a*) the no-substrate oxygen uptake of the brain preparation (unshaded bar to left of zero line), (*b*) the effect of morphine (0.12 per cent) on the no-substrate oxygen uptake (crosshatched bars to left of zero line), (*c*) extra oxygen uptake resulting when substrate supplement was added (unshaded bars to right of zero line), and (*d*) extra oxygen uptake in the presence of added substrate and 0.12 per cent morphine (shaded bars to right of zero line).

Reference to the data on the left of the zero line indicates that morphine has no characteristic effect on the no-substrate oxygen uptake of rat cerebrum irrespective of the type of preparation used. In the twenty-four experiments presented, a slight inhibition was observed nineteen times, whereas in five experiments the oxygen uptake was increased. Most of these changes are within the range of experimental error.

Experiments with minced cerebrum. Glucose, lactate, pyruvate, succinate, and α-ketoglutarate produce an appreciable increase in oxygen uptake when added to minced cerebrum. Only a slight increase in oxygen uptake is noted when fumarate and malate are added whereas no increase results from the addition of citrate.

Morphine inhibits the increase in oxygen uptake due to the addition of lactate by approximately 40 per cent. A slight reduction in the extra oxygen uptake in the presence of pyruvate and α-ketoglutarate is produced by morphine but this is of questionable significance. Morphine has no effect on the oxygen uptake of brain mince in the presence of added glucose, fumarate, malate or succinate.

Experiments with washed, homogenized cerebrum. The results obtained with washed, homogenized cerebrum when glucose, lactate, pyruvate, succinate, fumarate, malate, and citrate are added are essentially similar to those obtained with the minced tissue. The oxygen uptake of the homogenized preparation in the presence of added α-ketoglutarate is much reduced when compared with the minced and particularly the sliced tissue. Evidently homogenizing and washing, and even mincing to a lesser extent, remove some components necessary for the optimum oxidation of this substrate.

The extra oxygen uptake of washed, homogenized rat cerebrum produced by the addition of lactate or pyruvate is reduced about 20 per cent by morphine. In contrast with the effects in minced tissue, morphine reduces the extra oxygen uptake due to added glucose about 20 per cent. No significant morphine effect is observed with the other substrates used.

Experiments with cerebral cortex slices. The increase in the oxygen uptake of cortical slices resulting from the addition of glucose, lactate, pyruvate, α-ketoglutarate, succinate and fumarate is much greater than that which occurs with either minced or washed, homogenized tissue. Only a slight increase occurs with the addition of malate, none with citrate.

Morphine reduces by 10 to 30 per cent the extra oxygen uptake of sliced cortex in the presence of lactate pyruvate and α-ketoglutarate whereas this drug does not influence significantly the extra oxygen uptake which occurs with the addition of glucose succinate fumarate or malate

II. EFFECTS OF MORPHINE CODEINE AND THEBAINE ON CERTAIN DEHYDROGENASES

Procedure

The activity of five different dehydrogenases was determined in the presence of three different concentrations of morphine, codeine and thebaine The Thunberg technic was employed in all experiments The Thunberg tubes were evacuated and flushed out with nitrogen twice, evacuated again and placed in a water bath at 38°C until temperature equilibrium had been established before the methylene blue was tipped in Phosphate buffer was used throughout A cytochrome oxidase preparation of pig heart (9) was used as a source of succinic and lactic dehydrogenases Alcohol dehydrogenase was prepared from pig liver (10) as were citric (11) and glucose (12) dehydrogenases Morphine was used as the sulfate codeine as the phosphate and thebaine as the hydrochloride in concentrations of 0 06 0 12 and 0.24 per cent The coenzyme I used in all of these experiments was prepared from brewer's yeast according to the method of Ohlmeyer (13)

Most of these experiments were performed before it was recognized that dehydrogenases in the presence of coenzyme do not reduce methylene blue in the complete absence of diaphorase It is possible although not proven, that the relatively crude preparations of the coenzyme I-linked dehydrogenases used here may be deficient in this enzyme Since no diaphorase was added the exact quantitative effects of the three drugs are not demonstrated by these experiments Since qualitative results are considered to serve the purpose of the present survey we have not felt justified in repeating these experiments at this time in order to check this point. In the experiments involving the use of a more highly purified preparation of lactic dehydrogenase (14) diaphorase I prepared according to Straub (15) was added in adequate amounts

Results

Data of representative experiments are presented in figures 2, 3 and 4 Along the ordinates are plotted the times in minutes required for 80 per cent decolorization of the methylene blue Each bar represents the average time required for decolorization in duplicate tubes The unshaded bar represents the average control decolorization time and the shaded bars the average decolorization times in the presence of the various concentrations of the drug used in that particular experiment. Each experiment was repeated at least once and usually several times with the same or with a fresh enzyme preparation.

Lactic dehydrogenase Representative results of experiments involving the action of morphine codeine and thebaine on two types of lactic dehydrogenase preparations are presented in figure 2. Whereas the components of the systems of the two experiments (*1* and *2*—fig 2) in which the source of lactic

dehydrogenase was a preparation of pig heart, were qualitatively similar, quantitative differences in the concentrations of coenzyme I accounted for the wide difference in control decolorization times In experiment 1 and other similar experiments in which the decolorization times were short (ten minutes), all of the drugs used accelerated the velocity of decolorization from 35 to 94 per cent In experiment 2, in which the control decolorization time was over three hours, the percentage acceleration was even greater than with the shorter decolorization times

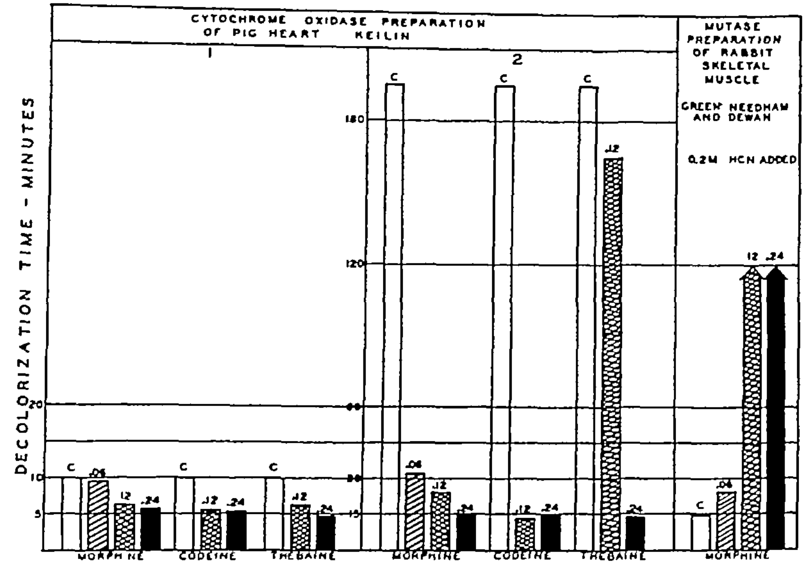

FIG 2 EFFECTS OF MORPHINE CODEINE AND THEBAINE ON THE ACTIVITY OF TWO DIFFERENT PREPARATIONS OF LACTIC DEHYDROGENASE

A Cytochrome oxidase preparation, 1 0 cc , coenzyme I (20 mgm /cc), 1 0 cc , *d*-Na lactate (M/10), 2 0 cc , methylene blue (0 05 per cent), 1 0 cc , phosphate buffer (M/60, pH 7 4), to 10 cc *B* Enzyme, 2 0 cc , coenzyme I (40 mgm /cc), 1 0 cc , flavoprotein, 1 0 cc , *dl*-Na lactate (2M), 0 4 cc , HCN (2M), 1 0 cc , methylene blue (0 01 per cent), 0 5 cc , water to 10 cc

In all of the experiments presented in figure 2, *d*- or *dl*-lactic acid served as the substrate In experiments not shown, but similar to experiments 1 and 2, figure 2, except for the use of *l*-lactic acid to replace the natural or racemic forms, no change was observed in the rate of decolorization of methylene blue These latter experiments indicate that *l*-lactic acid is not oxidized by this system, and that the increased rate of decolorization in the presence of morphine, codeine and thebaine obtained in experiments 1 and 2 is probably due, not to the dehydrogenation of these drugs, but to an effect by them on hydrogen transfer

In an attempt to relate the above results more specifically to lactic dehydrogenase other experiments with a more highly purified lactic dehydrogenase preparation were performed since it is known that the pig heart preparation contains several dehydrogenases The results of a characteristic experiment are shown in the last column of figure 2 Cyanide was added as a ketone fixative in order to inactivate the end product, pyruvate since this acid is known to inhibit the reaction. In the absence of cyanide no activity of the system was demonstrable When 0 06 per cent morphine was added to the active system an inhibition of activity of approximately 40 per cent occurred. The inhibition was complete with 0 12 and 0 24 per cent morphine

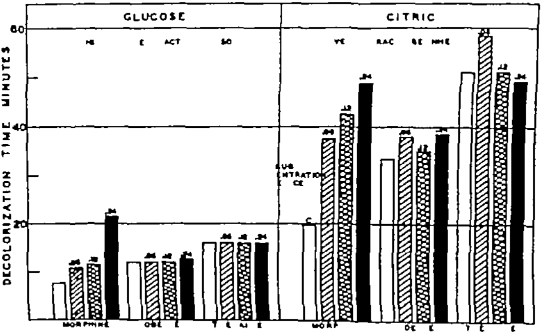

FIG 3. EFFECTS OF MORPHINE CODEINE AND THEBAINE ON THE ACTIVITY OF GLUCOSE AND CITRIC DEHYDROGENASES

Glucose dehydrogenase system Enzyme (50 mgm /cc) 1 0 cc. coenzyme I (20 mgm./cc) 1 0 cc., glucose (M/10) 2.0 cc. methylene blue (0 025 per cent) 1 0 cc phosphate buffer (M/60 pH 7 4) to 10 cc.

Citric dehydrogenase system Enzyme, 1 0 cc coenzyme I (20 mgm./cc.) 1 0 cc. Na citrate (M/10) 1.0 cc methylene blue (0.02 per cent) 1 0 cc phosphate buffer (M/10, pH 8.0) to 10 cc

Glucose dehydrogenase. Morphine in all concentrations inhibits the dehydrogenation of glucose by an extract of pig liver (fig 3) The inhibition ranges from 25 to 50 per cent with concentrations of morphine from 0 06 to 0.24 per cent. This system is not affected by either codeine or thebaine in similar concentrations

Citric dehydrogenase The dehydrogenation of citrate is inhibited 47 to 58 per cent with concentrations of morphine ranging from 0 06 to 0.24 per cent. No significant inhibition of this system is produced by codeine or thebaine

Succinic and alcohol dehydrogenases The three drugs, morphine, codeine and thebaine, in concentrations from 0 06 to 0 24 per cent, did not affect significantly the rate of dehydrogenation of succinic acid or alcohol by the dehydrogenase preparations employed (fig 4)

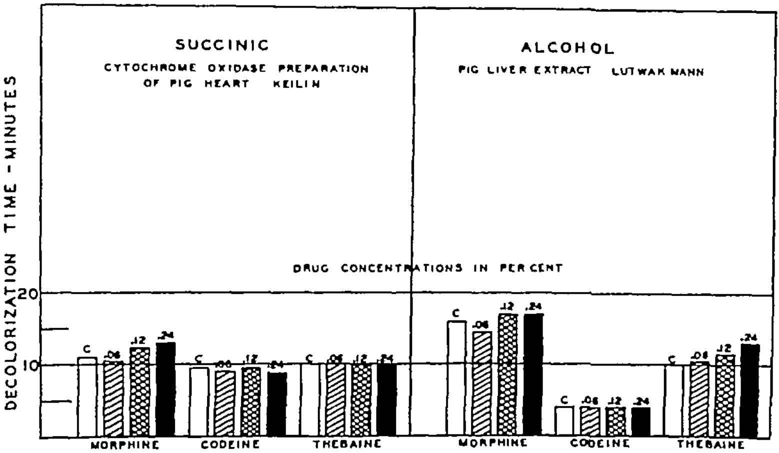

FIG 4 EFFECTS OF MORPHINE, CODEINE AND THEBAINE ON THE ACTIVITY OF SUCCINIC AND ALCOHOL DEHYDROGENASES

Succinic dehydrogenase system Enzyme, 1 0 cc , Na succinate (M/10), 1 0 cc , methylene blue (0 05 per cent), 1 0 cc , phosphate buffer (M/60, pH 7 4), to 10 cc

Alcohol dehydrogenase system Enzyme (80 mgm /cc), 1 0 cc , coenzyme I (20 mgm /cc), 1 0 cc alcohol (95 per cent), 0 1 cc , methylene blue (0 025 per cent), 1 0 cc , phosphate buffer (M/60, pH 7 4), to 10 cc

COMMENT

The observed reduction in oxygen uptake of rat cerebrum which results from the addition of morphine in the presence of certain added substrates confirms in a general way the results of Quastel and Wheatley (2) The significance of these results in relation to the effects of morphine on the intact organism remains an open question The concentrations of morphine used in these experiments is much higher than any which would be attained in the animal body Plant and Pierce (16) were able to recover 1 31 mgm of morphine per 100 grams of tissue from the central nervous system of non-tolerant dogs, four hours after the subcutaneous administration of 50 mgm per kgm , a concentration of only 0013 per cent We have purposely used these high concentrations of morphine in this survey for two reasons, (*a*) to allow us to compare our results with previous observations of others, and (*b*) to eliminate, if possible, certain systems from more detailed consideration in subsequent experiments, assuming that negative results obtained with high concentrations would probably hold for smaller quantities of the drug

The insignificant effect of morphine in a concentration one hundred times that which might be expected *in vivo* on the no-substrate oxygen uptake of rat cerebrum, inspires the question as to the validity of this type of approach to the basic problem of morphine action. It must be appreciated that the present results serve only as a point of departure for subsequent investigation and that the final unravelling of the action of morphine on oxidative processes involves not only determinations of total oxygen uptake but also knowledge of what substrates are affected (possibly including morphine itself) and what proportionate part of the total oxygen consumed is utilized for the oxidation of each individual substrate. Encouragement to continue investigations along these lines comes from the fact that a certain specificity of action of morphine can be demonstrated by utilizing the several dehydrogenase systems. We realize that the results in some instances may be misleading and that it is impossible at present to interpret them in relation to the action of the drug in the body

The marked increase in the rate of hydrogen transfer to methylene blue when morphine, codeine and thebaine are added to the phosphate extract of heart muscle used as a source of lactic dehydrogenase is of considerable interest The increase does not seem to be due to an accelerating effect of morphine on lactic dehydrogenase in view of the clear-cut inhibition which occurred with the more highly purified preparation. It is conceivable although it does not seem probable that morphine acts like cyanide to inactivate the pyruvate formed in this reaction thus inducing the disequilibrium necessary for the continued oxidation of lactate Since no hydrogen transfer to methylene blue occurs in this system in the presence of morphine when the non-oxidizable *l* lactic acid serves as the substrate it appears that morphine itself is not furnishing the H ions necessary for the rapid transfer which occurs when the *d* or *dl*-lactic acid serve as substrates. This same line of reasoning applies to the oxidation of other preformed substrates or in fact to other systems which may be present, unless it is assumed that the mere presence of an oxidizable form of lactic acid facilitates the oxidation of morphine or completes some other system It seems clear that the responsible system is coenzyme I-linked since no acceleration of hydrogen transfer is produced by morphine in the same heart muscle extract to which this coenzyme has not been added (succinic dehydrogenase system)

SUMMARY

The effects of morphine codeine and thebaine on several dehydrogenases prepared from animal tissues, and on the oxygen uptake of various preparations of rat cerebrum in the presence of certain added substrates have been determined.

Lactic citric and glucose dehydrogenases are inhibited by 0 06 0 12 and 0 24 per cent morphine Succinic and alcohol dehydrogenases are not affected.

Neither codeine nor thebaine in concentrations of 0 06, 0 12 and 0 24 per cent has any significant effect on glucose, succinic, alcohol or citric dehydrogenases Morphine, codeine and thebaine increase the rate of reduction of methylene blue by a phosphate extract of heart muscle

Morphine (0 12 per cent) has no significant effect on the no-substrate oxygen uptake of cerebral cortex slices, cerebral mince, or washed, homogenized cerebrum of the albino rat The oxygen uptake in the presence of added lactate is inhibited in all three preparations of cerebrum by 0 12 per cent morphine The oxygen uptake of cerebrum in the presence of added glucose, pyruvate and α-ketoglutarate is reduced by morphine in some preparations but not in others, whereas it is unaffected by morphine in the presence of added citrate, succinate, fumarate and malate

The authors are indebted to Drs F J Stare and V R. Potter for suggestions and criticisms

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EFFECTS OF MORPHINE AND ITS DERIVATIVES ON INTERMEDIARY METABOLISM[1]

II THE INFLUENCE OF THIAMIN DEFICIENCY ON THE RESPIRATION OF SKELETAL MUSCLE AND COCARBOXYLASE CONTENT OF TISSUES OF NORMAL AND CHRONICALLY MORPHINIZED RATS

F E SHIDEMAN AND M H SEEVERS

From the Department of Pharmacology University of Wisconsin, Madison

Received for publication November 18 1940

Fitzhugh (1) reported recently that thiamin relieves the preinjection irritability of rats chronically poisoned with morphine It seemed possible that observations dealing with the effects of morphine on the oxygen consumption of skeletal muscle (2) and cerebrum (3 4) in the presence of added pyruvate might be related to the findings of Fitzhugh in view of the established relationships between thiamin and pyruvate metabolism. If the increase in nervous irritability induced by chronic morphinism is specifically relieved by the administration of thiamin it is reasonable to expect that it would be increased by a thiamin deficiency We reasoned that the most likely action of morphine would be to affect either the enzyme concerned with the decarboxylation of pyruvic acid or its coenzyme cocarboxylase. If either of these was affected the tissues of animals chronically poisoned with morphine might be expected to demonstrate an altered capacity to oxidize pyruvate Such might be the case if the enzyme or coenzyme were either reduced in quantity or inactivated in some other manner

We therefore have made cocarboxylase assays of tissues and determinations of oxygen consumption of skeletal muscle from normal, thiamin-deficient, chronically morphinized, and thiamin-deficient morphinized rats. Irritability studies were also conducted to determine whether a concurrently induced thiamin deficiency increases the irritability of morphinized animals.

METHOD

The animals albino rats weighing between 100 and 200 grams were divided into five groups.

Group 1 (chronic morphine) comprised eighteen animals which received a modified

[1] Supported by the Wisconsin Alumni Research Foundation

The work presented in this paper is correlated with the program of the Committee on Drug Addiction of the National Research Council and allied agencies.

Steenbock diet, adequate in all respects These animals were injected subcutaneously with morphine sulfate once per day, starting with a dosage of 20 mgm per kilogram and increasing to 200 mgm per kilogram in the course of eight weeks

Group 2 (thiamin-deficient) comprised eighteen animals which received a thiamin-deficient diet (5) of the following composition sucrose 62 per cent, casein 20 per cent, Wesson salts 4 per cent, yeast 10 per cent, corn oil 4 per cent The casein and yeast were previously treated with sodium sulfite to destroy the thiamin present

Group 3 (chronic morphine and thiamin-deficient) comprised eighteen animals which received a thiamin-deficient diet and the same dosages of morphine as those in group 1

Group 4 (chronic codeine) comprised six animals on a normal Steenbock diet which received 10 mgm per kilogram of codeine phosphate daily by subcutaneous injection at the beginning of the experiment and increasing to 40 mgm per kgm in the course of eight weeks

Group 5 (chronic phenobarbital) comprised six animals on the normal Steenbock diet which received 46 mgm per kilogram of phenobarbital sodium per day subcutaneously

Groups 4 and 5 were included for a comparison of the effects of a compound closely related to morphine, codeine, and a non-specific depressant, phenobarbital

Six animals from each of groups 1, 2 and 3 were used for irritability determinations and biweekly observations were made according to the method devised by Barlow (6), modified by Eddy and Himmelsbach (7), and used recently by Fitzhugh These studies were continued six weeks before the animals were sacrificed for respiration studies When it was apparent that not all of the thiamin-deficient animals would survive this length of time, some were sacrificed earlier The animals were killed by decapitation, the muscle from the hind legs was removed and minced with a Latapie mincer, and respiration studies were made using the Warburg technic Approximately 200 mgm of tissue were placed in each flask containing 2 cc of a Ringer-M/60 phosphate solution of pH 7 4 and 0 002 M sodium succinate (8) When present, the final concentration of pyruvate was 0 02 M and of morphine 0 12 per cent In those flasks containing cocarboxylase, thiamin was also present The added amount of each of these substances was 10 micrograms The experiments were conducted at 38°C and readings were made every fifteen minutes Oxygen uptake is expressed as cubic millimeters per milligram dry weight of tissue

The remaining survivors in groups 1, 2 and 3, and all animals in groups 4 and 5, were used for cocarboxylase assay Quantitative estimation of cocarboxylase in brain, liver and muscle was made according to the assay method of Lipschitz, Potter and Elvehjem (9)

RESULTS

General observations

Irritability Determinations of irritability were made at the beginning of the experiment and at the end of two, four and six weeks The determinations on the animals receiving morphine were carried out eighteen to twenty-four hours *after* the last injection In figure 1, the average struggles per minute are plotted against time The values are the mean for each group

The decrease in irritability in thiamin-deficient animals is marked when determined by this method The decrease is apparent even from direct observations of general behavior We were unable to detect any increase in the pre-injection irritability of chronically morphinized rats receiving an adequate diet during eight weeks of poisoning, in fact, the curve shown in

figure 1 indicates a decrease in irritability which is not in harmony with the findings of Fitzhugh.

The method likewise failed to reveal any quantitative difference in irritability between the thiamin-deficient group and thiamin-deficient animals also receiving morphine although the irritability was reduced below the original level in both instances. It is difficult to accept the conclusion that the general irritability was the same in these two latter groups in view of the fact that foot-gnawing was observed in 75 per cent of the thiamin-deficient animals

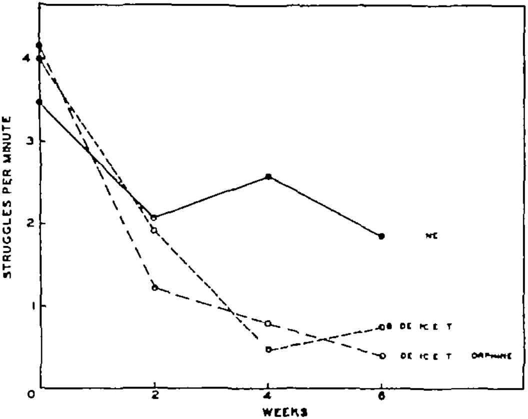

FIG 1 GROUP MEDIAN RESPONSES (STRUGGLES PER MINUTE) OF THIAMIN DEFICIENT RATS AND OF ADEQUATELY FED AND THIAMIN DEFICIENT RATS DURING THE DAILY ADMINISTRATION OF ASCENDING DOSES OF MORPHINE SULFATE

which received morphine This was noted within ten days after the initiation of the experiment and was so severe that in some cases the front feet were actually eaten away This behavior was not observed in a single thiamin-deficient animal which did not receive morphine. It was noted to a lesser degree however in a single animal receiving morphine which was on a normal diet

Weight Morphinized rats on an adequate diet exhibited a lag in weight gain the first week, followed thereafter by a progressive gain in weight which was less than normal Codeine and phenobarbital did not significantly affect the weight curves A gain in weight was observed in all of the thiamin

deficient animals during the first week but thereafter the loss in weight was marked until death or sacrifice, when the actual weight was 60 to 75 per cent of the original Chronic administration of morphine did not significantly modify the weight curve of thiamin-deficient animals

Length of life All animals receiving a normal diet lived the entire length of the experiment Four of the eighteen animals on the thiamin-deficient diet died thirty to thirty-five days after the beginning of the experiment The other animals in this group were sacrificed in not less than thirty-one days In the group of animals on a thiamin-deficient diet and also chronically poisoned with morphine, nine died between twenty-four and twenty-nine days after the beginning of the experiment The remaining animals were killed after the twenty-ninth day

TABLE 1

Mean values for no-substrate Q_{O_2} and mean percentage increases in oxygen uptake produced by the addition of various supplements to minced skeletal muscle from normal, chronically morphinized, thiamin deficient, and chronically morphinized-thiamin deficient rats

	MEAN Q_{O_2}	MEAN PERCENTAGE INCREASE IN OXYGEN UPTAKE PER MILLIGRAM DRY WEIGHT				
		Morphine	Pyruvate	Pyruvate morphine	Pyruvate cocarboxylase	Pyruvate morphine cocarboxylase
Normal	1 42	27	76	152	103	122
Group 1 chronic morphine	2 07	25	30	39	32	61
Group 2 thiamin deficient	1 45	21	18	19	24	26
Group 3 chronic morphine thiamin deficient	1 79	18	19	12	15	32

Effect of morphine on oxygen consumption of skeletal muscle The mean values of no-substrate Q_{O_2} of respiring minced skeletal muscle of the four groups of rats are presented in table 1 The increases in oxygen uptake produced by the addition of various supplements are likewise shown in this table and are calculated in terms of the percentage increase above the no-substrate Q_{O_2} for each group The latter figures represent the means of at least two satisfactory experiments Representative curves for the effects of supplements added to the muscle of rats in each group are shown in figures 2 and 3 Each set of curves is characteristic for all experiments in that group

An examination of the mean values for no-substrate Q_{O_2} in the four groups (column 1, table 1) reveals a higher value in the animals chronically poisoned with morphine Whereas this finding was consistent and highly suggestive, insufficient data are as yet available to prove conclusively that the skeletal muscle of chronically morphinized animals respires at a higher level *in vitro* Experiments are in progress to determine this point

Morphine (0 12 per cent), in the absence of other supplements, produces an

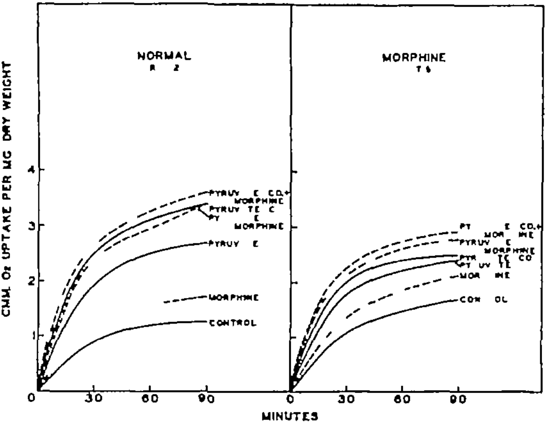

FIG. 2. INDIVIDUAL CURVES DEMONSTRATING THE EFFECTS OF VARIOUS SUPPLEMENTS ON THE OXYGEN UPTAKE OF SKELETAL MUSCLE FROM ADEQUATELY FED RATS AND FROM ADEQUATELY FED RATS FOLLOWING THE DAILY ADMINISTRATION OF ASCENDING DOSES OF MORPHINE SULFATE

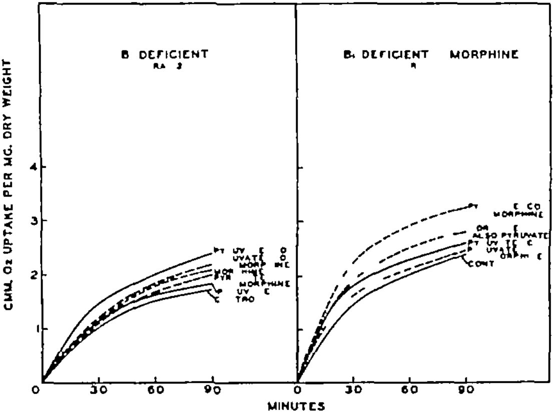

FIG. 3. INDIVIDUAL CURVES DEMONSTRATING THE EFFECTS OF VARIOUS SUPPLEMENTS ON THE OXYGEN UPTAKE OF SKELETAL MUSCLE FROM THIAMIN DEFICIENT RATS AND FROM THIAMIN DEFICIENT RATS FOLLOWING THE DAILY ADMINISTRATION OF ASCENDING DOSES OF MORPHINE SULFATE.

increase in oxygen uptake of skeletal muscle ranging from 18 to 27 per cent in all of these four groups This may be observed readily in figures 2 and 3 A concentration of sodium sulfate containing an amount of sulfate ion equal to that in the quantity of morphine sulfate used produced no significant effect Much smaller concentrations of morphine are effective in producing this increase in normal rat skeletal muscle, as is indicated from the representative experiment shown in table 2

Pyruvate as a supplement produces a mean increase of 76 per cent in the oxygen uptake of normal muscle whereas the increase in the muscle from chronically morphinized animals is only 30 per cent and is less than 20 per cent in all animals of both groups on the thiamin-deficient diet

Pyruvate and morphine are synergistic in their effect when both are added to normal muscle It is evident that the 152 per cent increase produced by pyruvate and morphine together is greater than the 27 per cent increase produced by morphine and the 76 per cent increase due to pyruvate when each

TABLE 2

Effect of morphine sulfate on Q_{O_2} of normal rat skeletal muscle

MORPHINE	Q_{O_2}	CHANGE
per cent		*per cent*
None	1 71	
005	1 99	+16
01	1 91	+12
05	2 18	+28
1	2 50	+46
5	2 55	+49

supplement is added singly This synergistic action is lacking in the chronically morphinized or thiamin-deficient muscle, in fact, the addition of the two supplements, morphine and pyruvate, produces little more effect than either added singly, so that the results are not even additive

Addition of cocarboxylase increases the oxygen uptake in the presence of added pyruvate by normal muscle mince but this effect is not produced in chronically morphinized or thiamin-deficient muscle

The effects of morphine, pyruvate and cocarboxylase, when present together, appear to be additive except in muscle from thiamin-deficient animals

Cocarboxylase assays

A summary of the results of cocarboxylase determinations on brain, liver and muscle from animals of the various groups is presented in figure 4 Each point is the average of duplicate determinations on the extract of a given tissue and is expressed as micrograms of cocarboxylase per gram of wet tissue The

solid lines are the means for each tissue The assay values for cocarboxylase in the brain, liver and muscle of normal animals (column 1) are in harmony with those reported by other investigators (10) Chronic poisoning with morphine codeine or phenobarbital of animals on an adequate diet does not significantly modify these levels.

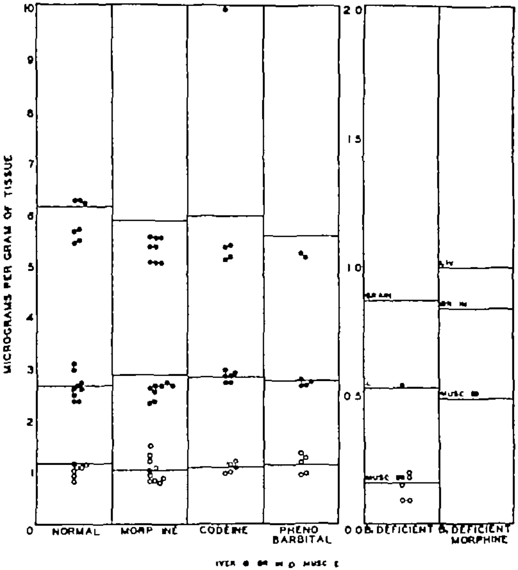

Fig 4 Cocarboxylase Content of Brain Liver and Muscle from Normal and Thiamin Deficient Rats, Rats Chronically Poisoned with Morphine Codeine or Phenobarbital and Thiamin Deficient Rats Chronically Poisoned with Morphine

The cocarboxylase content of all tissues examined of the two groups of animals on a thiamin-deficient diet was greatly reduced. These data are presented in the last two columns of figure 4 The mean values for brain cocarboxylase were reduced to approximately 30 per cent of normal in the

thiamin-deficient and thiamin-deficient—chronically morphinized animals An even greater reduction was observed in liver and muscle in these two groups The mean values for liver and muscle are higher in those animals also receiving morphine No significance is attached to these latter findings, however, in view of the small number of determinations, the wide individual variation, and the possible differences in water and fat content of these tissues Since the absolute quantities of cocarboxylase in these tissues are so small, expression on a wet weight basis may lead to considerable error

COMMENT

We were unable to establish the occurrence of pre-injection irritability in the rat during chronic morphine poisoning of eight weeks duration by the objective struggle method Complete thiamin deficiency decreased rather than increased the irritability Whether an increase in irritability could be established by chronic thiamin deficiency of a lesser grade over a longer addiction period should be established. The foot-gnawing observed in the chronically morphinized—thiamin-deficient animals and the shorter life span of these animals would indicate that either condition may be aggravated by the existence of the other Whereas these results do not support the view that all signs of morphine withdrawal are due to a thiamin deficiency, they do not deny a possible aggravation of such signs by a coexistent vitamin deficiency Himmelsbach (11) was unable to control or even significantly affect the withdrawal syndrome in man with massive doses of thiamin

The results of the cocarboxylase assays seem to indicate that no thiamin deficiency existed in these chronically morphinized animals although the similarities in the response of chronically morphinized and thiamin-deficient muscle to pyruvate and cocarboxylase added *in vitro* might suggest, although not prove, that a metabolic defect exists which is common to both conditions

The increase in oxygen consumption resulting from the addition of morphine to normal rat skeletal muscle could be due to any one or combination of numerous factors, among which might be suggested (*a*) the oxidation of morphine, (*b*) liberation of substrate which is subsequently oxidized, (*c*) an accelerating effect of morphine on the enzymatic oxidation of preformed substrate, (*d*) the inactivation by morphine of some substance which normally inhibits oxidations The data presented here do not lend conclusive support to any one of these suggested possibilities, although some evidence is obtained which might indicate that morphine increases the oxidation of pyruvate We are now engaged in studies involving determinations of the rate of disappearance of this substrate Similar methods will be required to discover whether morphine is oxidized under these circumstances If the latter is the case, it is not proven from the results obtained thus far, since no quantitative relationship has been established between the amount of extra oxygen uptake and the quantity of morphine present

The smallest concentration of morphine sulfate that we have used (005 per cent) produces a significant increase in the oxygen uptake of normal rat skeletal muscle This is not an unreasonable concentration to expect *in vivo* during the administration of large doses of the drug if we may be allowed to draw deductions from the results of previous studies Wolff, Riegel and Fry (12) recovered 36 mgm. of morphine per 100 grams of skeletal muscle from a dog which had received 1 11 grams per kilogram intravenously during a five-hour period If these quantitative relationships are assumed, a concentration of 1 6 mgm per 100 grams of muscle should have been present had 50 mgm. per kilogram been administered a concentration of 0016 per cent. Plant and Pierce (13) actually found a concentration of 0015 per cent morphine in the skeletal muscle of a non tolerant dog four hours after the subcutaneous administration of 50 mgm per kgm. The uniform findings of an increase in oxygen uptake when morphine is added to respiring rat skeletal muscle the accelerating effect of morphine on the hydrogen-donating activity of a phosphate extract of heart muscle (4) and the suggestive, although as yet not conclusive finding of a considerably greater no-substrate oxygen consumption of chronically morphinized rat muscle in comparison to normal seem to justify a certain amount of speculation regarding the possible relationships of these observations to chronic morphinism Assuming on the basis of the previous discussion that a concentration of morphine equal to the lowest used in the present experiments (005 per cent) may be obtained in the animal body and taking cognizance of the fact that a large portion of the morphine which may be recovered from the body is found in skeletal muscle (Wolff Riegel and Fry administered 11.33 grams and recovered 4 48 grams 2.3 grams of which was from skeletal muscle Plant and Pierce found 80 per cent of the total quantity which they recovered from tissues to be in skeletal muscle), it is then reasonable to advance as a working hypothesis that this morphine may be exerting a sustained and reasonably uniform accelerating action on oxidations in muscle either independently of or in conjunction with nervous influences. Furthermore it could be postulated that this localized increase in metabolism is not apparent during continued administration of the drug due to the masking depressant effect of morphine on the cerebrum. Since skeletal muscle represents such a large portion of the actively respiring tissue in the body a significant increase in total metabolism of the animal should be evident when the central effects of the drug have abated as in withdrawal providing the local action in muscle is of considerably greater duration than the central effect. Plant and Slaughter (14) and Barbour Porter and Seelye (15) have shown that such an increase in total respiratory metabolism does occur in the dog after the principal depressant effects have subsided and before any increase in activity of the animal is measurable. The evidence at hand does not warrant a long discussion of the possible implications of such a suggestive hypothesis as it relates to tolerance

and to the signs and symptoms of addiction Nevertheless, certain facts such as the greater oxygen unsaturation of venous blood in human addicts during withdrawal (16), the rapid loss of weight in thyroid-fed animals chronically poisoned with morphine (17), and many others, could be easily harmonized with such a view Obviously, such an hypothesis would have to be correlated with the possibility of simultaneous alterations in metabolism in other tissues, and increases in activity of certain nervous structures, such as the spinal cord (2)

SUMMARY

Severe thiamin deficiency was induced concurrently with chronic morphine poisoning in albino rats Although the animals in this group exhibited foot-gnawing and succumbed earlier than the thiamin-deficient controls, no increase in the pre-injection irritability was obtained, either in this group or in the control groups, by the objective struggle method

The addition of either morphine or pyruvate results in a marked increase in the no-substrate oxygen uptake of normal rat skeletal muscle When both are added together, the resultant increase is greater than that which would be expected from summation of the two individual effects

The extra oxygen uptake resulting from the addition of pyruvate to chronically morphinized rat skeletal muscle is less than half of that which occurs in normal muscle, and the synergistic increase noted in normal muscle when morphine and pyruvate are added is lacking Results of a similar and even more striking nature were obtained with thiamin-deficient muscle

The increase in the oxygen uptake of normal muscle resulting from the addition of cocarboxylase in the presence of added pyruvate was entirely lacking in both thiamin-deficient and chronically morphinized muscle

The cocarboxylase content of brain, liver and skeletal muscle is not changed significantly by chronic poisoning with morphine, codeine or phenobarbital Neither does the chronic administration of morphine modify significantly the degree of reduction of cocarboxylase induced by thiamin deficiency

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THE ACTION OF VITAMIN C AND PHENOL OXIDASE IN THE INACTIVATION OF BETA PHENYLISOPROPYLAMINE (AMPHETAMINE)[1]

KARL H. BEYER

The Department of Physiology, University of Wisconsin Medical School, Madison

Received for publication January 22, 1941

In an earlier communication we reported that about half a given dose of amphetamine was excreted as such by the kidneys over a period of 48 hours (1) We found that this figure was independent of the mode of administration and was not due to excretion of the drug in a conjugated form or to the oxidation of one or the other of the optically active forms Later (2) we reported that the drug was one of a group of phenylisopropylamines not oxidized by either amine oxidase or phenol oxidase However, if liver function was impaired, the excretion of the drug became as much as 100 per cent of a given dose (1)

These observations led us to conclude that benzedrine (amphetamine) was slowly and partially inactivated in the body The purpose of this research was to investigate how this inactivation might be carried out

PROCEDURE

This work was divided into two phases In the first we undertook to determine *in vitro* whether the reactions described here took place, in the second the significance *in vivo* of one of these processes was investigated

Extracts containing phenol oxidase were prepared from potatoes according to the method previously described (2) We found this extract to be satisfactory for our work and much more simply prepared than the more highly purified enzymes of Kubowitz (3), and Keilin and Mann (4)

The amount of ammonia given off was estimated by the following method Two 150 cc test tubes closed by 2 hole rubber stoppers were connected in series by glass tubing This was done in such a manner that air drawn through the tubes could be caused to bubble through solutions contained in both tubes The first of the two tubes was clamped submerged to its rim in a water bath designed to hold six units and maintained at 37°C The second tube remained outside the bath The first tube contained the enzyme or ascorbic acid, amphetamine and buffer, the second contained 40 cc of N/20 HCl Air drawn through the solution in the tube served to oxygenate and agitate the system The ammonia given off during the reaction was drawn over into the HCl in the second tube The amount of ammonia dissolved in the HCl was calculated by micro-titrating the excess HCl with NaOH against methyl orange as an indicator

[1] Made possible in part by a grant from the Wisconsin Alumni Research Foundation

The HCl and NaOH solutions were standardized against Na_2CO_3. The bath rested on two electric hot plates which could be used to elevate the temperature of the water when desired.

Experiments were allowed to run for periods ranging from 18 to 24 hours at the end of which time 5 cc of 10 per cent NaOH was added to each tube in the bath and the water brought to 85°C for 3 hours to distill over any ammonia remaining in solution. The tests and controls were set up in duplicate The difference in the average titration values for tests and controls was used to calculate the amount of ammonia attributable to deamination

For the *in vivo* experiments 5 dogs were put in metabolism cages so that their total urine volume per 24 hours might be collected Ten milligrams of benzedrine sulfate (calculated as the amine) were injected daily beneath the loose skin of the neck The excretion of the drug was followed for at least 4 days or until the animal was saturated and the output of the amine in the urine was fairly constant.

Following this initial saturation period ascorbic acid plus half its weight of $NaHCO_3$ was injected daily Dogs A and B received 200 mgm. of ascorbic acid and 100 mgm of $NaHCO_3$ and the others 400 mgm. of vitamin C and 200 mgm of $NaHCO_3$ per day The chemicals were dissolved in 10 cc of distilled water and immediately injected into the flank of the dogs, the total volume being divided into 2 or 3 injections To be sure that the $NaHCO_3$ was not influencing the results in dogs C and D the injection of the base was continued after the vitamin had been stopped In dog E the $NaHCO_3$ injections were started at the beginning of the experiment and stopped when the ascorbic acid injections were discontinued.

The method for estimation of amphetamine was previously reported by us (1) However the procedure was slightly modified for this work Instead of 0.2 grams of MgO per aliquot of urine we have found 40 to 50 mgm. portions of MgO sufficient. With these smaller amounts the recovery of a known amount of the amine as compared with the same amount in aqueous solution not taken through the adsorption-extraction procedure, was 100 per cent. Five cc portions for approximately each 200 cc of the total 24 hour urine volume gave satisfactory aliquots

We have noted that the temperature at which the coupling reaction occurs has an appreciable influence on the intensity of the color produced Coupling at the generally available controlled temperature of 37°C gives a more uniform color of greater intensity than when done at room temperature Following coupling the solutions are removed from the incubator room and the base added. We add the 5 cc of 0 7 per cent Na_2CO_3 to the coupled product slowly allow the solution to stand for 15 minutes and then add the 1 cc of 10 per cent NaOH drop by drop to produce the red color Rapid addition of the NaOH with the omission of the Na_2CO_3 inhibits production of the red color

The colored solution resulting from the coupling of the amine with para nitrobenzene-diazonium chloride was extracted with 15 cc of *n* butanol Instead of waiting until the butanolic extract was absolutely clear which might take some time if the separatory funnel is vigorously shaken about 15 minutes after the extraction 10 cc of the butanol was pipetted into the photocolorimeter tube to which was added 0.5 cc of *iso*-propanol This gave a perfectly clear solution immediately and was much quicker than the older method Concentrations from 0 01 to 0 1 mgm. amine when plotted against the galvanometric deflection of the corresponding color intensities using an Evelyn Photoelectric Colorimeter gave a curve of the formula

$$x = a - b \log y$$

where x = concentration in milligrams, y = galvanometer deflection in cms. a = 0 103 and b = 0 110

Unless one is very careful to observe the precautions given above there may occasionally be deviations of a 0 1 mgm control from the calculated value If the Duboscq Colorimeter and serial standards made up at the time of the test be used, this deviation does not disturb the results Where the deviation of the control is not great a linear correction to the curve introduces an insignificant error

EXPERIMENTAL RESULTS

Deamination of phenylisopropylamine activated by phenol oxidase

In another communication (2) we reported that in the oxidation of paredrine (*p*-hydroxyphenylisopropylamine) in the presence of phenol oxidase deamination of the molecule occurred We concluded that the *o*-quinone formed in the ring of paredrine in one stage of its oxidation was capable of oxidizing the amino group on the side chain to the corresponding imino form, at the

TABLE 1

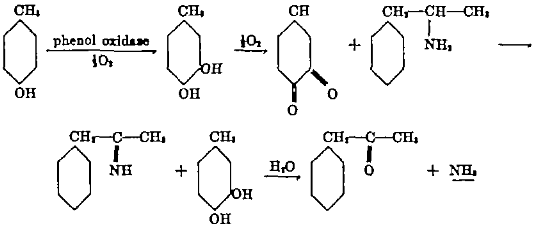
The deamination of amphetamine sulfate by the phenol oxidase p-cresol system

AMPHETAMINE SULFATE	*p*-CRESOL	THEORETICAL RECOVERY OF NH_3	AMMONIA RECOVERED	NH_3 RECOVERED
mgm	*mgm*	*mgm*	*mgm*	*per cent*
50	25	4 6	1 38	30 1
50	25	4 6	1 37	29 8
75	25	6 9	0 87	12 5
75	25	6 9	0 78	11 3
100	50	9 2	1 98	18 3
100	50	9 2	1 87	17 2
100	50	9 2	1 23	11 3
100	25	9 2	1 35	14 7
100	50	9 2	4 05	44 0
100	100	9 2	5 05	54 8

same time reducing the quinone to the *o*-dihydric compound In the presence of water the imino radical was replaced by oxygen, ammonia being liberated The *o*-dihydric phenyl group was simultaneously oxidized again to the *o*-quinone state Happold and Raper (5), Robinson and McCance (6), Hubard (7) and Pugh and Raper (8) found that in the presence of *p*-cresol, phenol or homoquinone phenol oxidase was capable of oxidizing glycine, phenylalanine and leucine with the liberation of ammonia Apparently the reaction was similar in the several instances

It seemed possible then, that in the presence of such compounds and phenol oxidase amphetamine might be deaminated To test this hypothesis we used the method for measuring ammonia production described above In the control tubes were contained 10 cc of phenol oxidase extract, *p*-cresol and 30 cc of M/20 phosphate buffer, pH 7 0 These tests were set up similarly with the addition of a weighed amount of benzedrine sulfate added in solution in the buffer

From table 1 it may be seen that deamination did occur and that by varying the concentrations of amphetamine and *p*-cresol we could account for 11.3 to 54.8 per cent of the theoretical amount of ammonia, assuming complete deamination of amphetamine and recovery of the ammonia It is probable that deamination was not complete or that some of the ammonia might have reacted with other substances such as aldehydes present in the phenol oxidase preparation The following equation is suggested for the reactions leading to the production of ammonia.



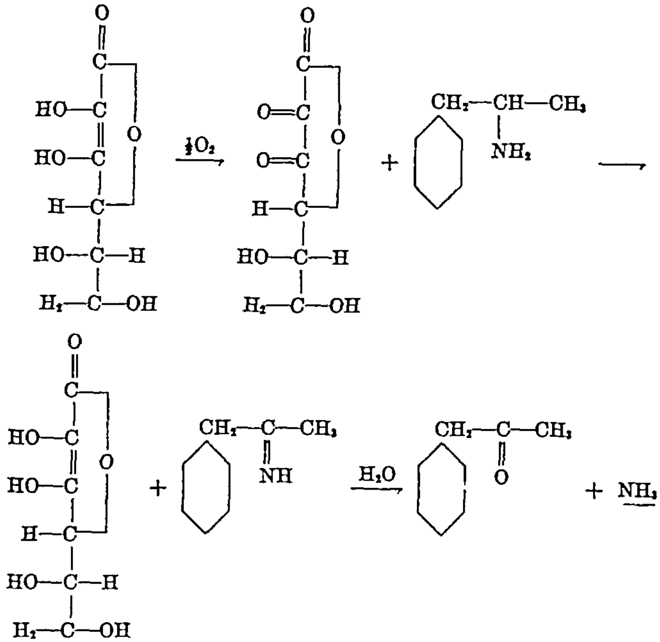
It is possible that H_2O_2 is formed in the course of the reaction, but this experiment permits no conclusion as to its place in the equation.

Whether this type of deamination could occur in the body is not certain. The probability of its occurrence is just as great as that of the formation of melanins in the body by the oxidation of tyrosine activated by phenol oxidase (tyrosinase) In the formation of melanins from tyrosine as presented by Evans and Raper (9) and in the review by Raper (10) *o*-quinones are formed which might act as hydrogen acceptors. However the physiological significance of phenol oxidase in mammalian tissues is still uncertain (11) though Block (12) Pugh (13) Charles (14) and Charles and Rowles (15) have reported tyrosinase reaction of tissue from mammals and birds

Deamination of amphetamine in vitro by ascorbic acid

Since 1937 Abderhalden has reiterated that ascorbic acid was capable of deaminating certain amino acids with the production of NH_3 CO_2 and the corresponding aldehydes (16) Earlier Euler Karrer and Zehender (17) observed that dehydroascorbic acid was capable of deaminating leucine with the formation of strongly reducing aldehydes or ketones Holtz also demonstrated that ascorbic acid was capable of deaminating amino acids and of decomposing iminazoles and purines *in vitro* (18 19) From these and other observations of our own it seemed possible that ascorbic acid oxidized to dehydroascorbic acid might play a rôle in the inactivation of amphetamine by

deamination The stages in the reaction involving inactivation could be written as follows



The method used for measuring deamination was that described in the Procedure The solutions were buffered at pH 7 0 using 40 cc of M/40 phosphate buffer The following table 2 summarizes a group of experiments estimating the amount of ammonia produced during the reaction This amounted to about 30 to 40 per cent of the theoretical production, assuming that deamination went to completion. However, it is unlikely that the reaction goes to completion only as indicated This is probably only one of the reactions that may be involved For example, Parrod (20) has reported that the oxidation of ascorbic acid in the presence of primary amines, oxygen and an alkaline medium gives rise to a series of oxamides and N-substituted analogues Some of these products may be responsible for the golden yellow color that tints the solution containing ascorbic acid and benzedrine when air is bubbled through

Whether ascorbic acid exists in the body in an oxidized form that would be necessary for these reactions has been disputed Basis for argument that dehydroascorbic acid does not exist in the body are the *in vitro* experiments which show that solutions of ascorbic acid are stabilized for the most part by an excess of compounds having a sulfhydryl group which is capable

of undergoing oxidation and reduction, i.e glutathione and cystine However, the experiments of Stotz, Harrer Schultze and King (21) demonstrated that a slow oxidation of added vitamin C did occur in guinea pig liver brei. The nature of the reaction and the effects of inhibitors indicated that the cytochrome-cytochrome oxidase system was involved. The glutathione naturally present in liver brei was unable to protect added ascorbic acid from oxidation. That the cytochrome-cytochrome oxidase is capable of oxidizing ascorbic acid has also been reported by Keilin and Hartree (22) and has been observed by us.

TABLE 2

The deamination of amphetamine sulfate by the dehydroascorbic-ascorbic acid system

AMPHETAMINE SULFATE	ASCORBIC ACID	THEORETICAL RECOVERY OF NH_3	AMMONIA RECOVERED	NH RECOVERED
mgm.	*mgm.*	*mgm.*	*mgm.*	*per cent*
50	50	4 6	2 42	52 7
50	100	4 6	2 55	55 5
50	100	4 6	1 48	32 2
75	60	6 9	2 48	36 0
75	60	6 9	1 66	24 0
75	150	6 9	2 26	33 0
100	30	9 2	2 52	27 6
100	100	9 2	4 40	48 0
100	100	9 2	2 94	32 0
100	100	9 2	2 61	28 4
100	100	9 2	3 56	38 8

The in vivo inactivation of amphetamine by ascorbic acid

Interesting as are the *in vitro* experiments with ascorbic acid deamination of amphetamine the physiological significance of such a relationship between the two compounds can only be evaluated by animal experimentation For this reason we turned to experimentation on dogs for a solution of the problem The methods used in this part of the experiment have been described in the Procedure

The results on five dogs are given in table 3 It is apparent that in every instance following saturation of the dog and establishment of control excretion levels for the drug, the administration of vitamin C reduced the excretion of the drug to about 35 per cent of the control levels. This reduction was always delayed and gradually became more apparent as the dogs were saturated with the vitamin Withdrawal of ascorbic acid caused the excretion of the amine to regain its control level in every instance except one Presumably in the exceptional instance not enough time was allowed for complete recovery Administration of the vitamin did not demonstrably result in conjugation of the amine since acid hydrolysis of the urine did not

TABLE 3

The effect of ascorbic acid on the daily excretion level of amphetamine

Dogs A and B received 200 mgm ascorbic acid (C) + 100 mgm $NaHCO_3$ Dogs C, D, and E received 400 mgm ascorbic acid + 200 mgm $NaHCO_3$

24 hour periods	Urine volume per 24 hours (cc.)	Mgm excreted per 24 hours	24 hour periods	Urine volume per 24 hours (cc)	Mgm excreted per 24 hours	24 hour periods	Urine volume per 24 hours (cc)	Mgm excreted per 24 hours
Dog A			Dog B			Dog C		
3rd	730	5 11	2nd	570	4 86	3rd	575	4 26
4th	300	3 00	3rd	530	4 11	5th	660	5 70
5th	365	3 65	4th	540	4 20	7th	375	6 18
Began C + $NaHCO_3$ on 5th day						Began C + $NaHCO_3$ 7th day		
12th	1025	0 90	12th	235	1 04	11th	560	2 39
13th	800	1 42	14th	245	2 20	12th	280	2 59
14th	450	1 48	15th	225	2 25	13th	650	1 82
Stopped C + $NaHCO_3$ on 16th day						Stopped C on 12th day		
19th	775	2 94	20th	450	3 54	15th	400	3 42
20th	845	4 70	22nd	625	4 10	16th	875	4 20
Dog D						Dog E		
						Started $NaHCO_3$ with experiment		
3rd	770	4 63				2nd	675	3 38
5th	1025	5 02				4th	405	3 86
7th	510	6 22				7th	240	2 83
Began C + $NaHCO_3$ on 6th day						Began C on 6th day		
9th	1005	2 51				11th	135	0 96
10th	980	2 32				12th	460	0 94
11th	370	1 93				13th	305	0 98
Stopped C on 11th day						Stopped C + $NaHCO_3$ on 13th day		
13th	870	4 70				14th	335	1 04
14th	455	3 94				15th	340	1 02

increase the recovery of amphetamine The $NaHCO_3$ acted only to neutralize the ascorbic acid solution and can be seen to have no effect on the experiment

SUMMARY

That amphetamine is capable of undergoing inactivation in the body and the means whereby this may be accomplished have been described

Through the intermediation of a monohydric phenolic compound (p-cresol) phenol oxidase has been shown to be capable of activating the deamination of beta phenylisopropylamine.

Ascorbic acid may play a rôle in the inactivation of amphetamine. By means of *in vitro* experiments it was shown that the drug was deaminated in the presence of ascorbic acid and oxygen buffered at pH 7 0 In dogs the daily administration of 200 or 400 mgm. of vitamin C reduced the excretion of the amine to about 35 per cent of the control output of the animals. In these experiments then the excretion of the drug was dependent at least in part on the vitamin C content of the animals. As the ascorbic acid content was increased and more of it made available for inactivation purposes the excretion of the amine was diminished As the ascorbic acid level of the animal subsided the excretion of amphetamine by the kidney increased to the control levels.

The author wishes to acknowledge his indebtedness to the Smith Kline and French Laboratories for a grant making possible for this work the technical assistance of Mr W Vernon Lee and to Dr Walter J Meek for his criticism of the work and this paper

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